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Configurationally Imprinted Biomimetic Polymers with Specific Recognition for Oligopeptides

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Configurationally Imprinted Biomimetic Polymers with Specific Recognition for Oligopeptides

by

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To My Loving Family and Friends,
For Your Constant Support, Understanding, and Patience.

Configurationally Imprinted Biomimetic Polymers with Specific Recognition of Oligopeptides

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The over-expression of several peptides and proteins in the body often leads to catastrophic physiological conditions. Ultimately it would be beneficial to be able to reduce the circulation of these peptides. To achieve this, we created systems that use synthetic biomaterials to mimic natural biological recognition processes. These recognitive polymer systems can be fabricated with molecular architectures possessing specific chemical moieties that provide a framework for selective recognition of a target analyte in aqueous environments. We concentrate on a particular peptide, angiotensin II, which would benefit from a system such as this. This work reports on a novel recognitive system that

recognizes and captures the undesirable analyte. To achieve destruction of the over-expressed peptide for further therapeutic effects, we have incorporated biodegradable components into the polymer backbone which create an acidic microenvironment, capable of destroying the peptide, upon hydrolytic cleavage at the ester bond.

Imprinted polymer networks were prepared by UV-initiated, free radical polymerization reactions of acrylamide as the functional monomer, poly(ethylene glycol dimethacrylate) as the crosslinking agent, and angiotensin II as the template molecule. To analyze the effectiveness of the imprinting process, recognitive/binding studies using angiotensin II and its derivative SVA angiotensin were conducted by HPLC. In order to optimize the repeated recognition (rebinding) of angiotensin II, the molar ratio of template to functional monomer was varied from 1:8, 1:16 to 1:32. The cross-linking ratio was also varied from 10% to 80%. The angiotensin II was then placed in various acid compositions and analyzed by mass spectroscopy in order to determine its integrity in the presence of an acidic microenvironment. The recognition studies showed that the networks imprinted for angiotensin II were more selective and recognized angiotensin II more effectively than the non-imprinted polymers and was more selective for angiotensin II than its derivative peptide SVA angiotensin. It can also be seen from studies that the peptide angiotensin II can be degraded in the presence of glycolic acid. The synergistic effect of the recognition,

capturing and destruction of the peptide ultimately offers promise for novel drug delivery systems.

TABLE OF CONTENTS

1 INTRODUCTION

In the past few decades there has been a major shift in research to develop drug delivery systems (DDS) that are able to respond directly to a person's individual needs as opposed to responding generically to groups of individuals diagnosed with a specific medical condition. Systems of this nature are able to maximize the efficacy of therapeutic agents by rapidly detecting disease states and often unnoticeable symptoms, thereby improving the treatment of the patient's medical condition as well as quality of life. This new field is known as "intelligent therapeutics" and involves responsive devices and drug delivery systems that can detect, capture, isolate and treat undesirable biologicals and trigger an action that will release drugs, proteins or therapeutic agents. To achieve such goals, one needs to be able to interface synthetic and hybrid materials with dynamic biological systems. One promising development in this field is the use of molecularly imprinted polymers (MIP).

The expanding field of molecular imprinting technology has an enormous potential to create intelligent, analyte-sensitive polymers that are capable of controlled drug delivery of therapeutic molecules in response to a biological event. MIP systems have received widespread attention as a new class of drug

delivery systems, and as diagnostic tools in molecular sensors in the treatment of disease.

MIPs have a large potential for use as DDS whether as the rate-limiting mechanism in controlled release systems, as the trigger for the release of a therapeutic compound in response to external stimuli or even as the sensing element to give feedback as part of a biological sensor. Our lab in particular has developed a specific molecular imprinting method termed “configurational biomimesis” which produces polymeric surfaces or polymeric recognitive networks that have three dimensional, stereospecific binding micro- and nanocavities based on a given template molecule. The new synthetic recognitive biomaterials have been designed to mimic biological recognition, which is ultimately an improvement over using expensive and unstable naturally occurring biological macromolecules and ligands.

Configurational biomimesis involves placing a template molecule in the presence of unreacted monomer and a crosslinker. These components need to be mutually soluble in an inert solvent. It is then possible to introduce an initiator which allows a free-radical polymerization to take place. This polymerization produces a mesh-like network which forms hydrogen bonding complexes with the opposing functionalities from the functional monomers and the template. The hydrogen bonding can then be disrupted and the template can be removed from the

network. The polymer is then left with three dimensional cavities containing free functionalities. Ultimately these cavities can reform hydrogen bond complexes when contact is reinitiated by the template molecule. The use of configurationally imprinted biomimetic polymers (CIBPs) as therapeutic agents holds particular promise for treating certain medical conditions. An example of such a condition is the overexpression of certain peptides or proteins in humans.

The concentration of peptide hormones and some proteins rhythmically fluctuate as a function of time. Most endocrine rhythms exhibit high-amplitude circadian ("about 24 hour") rhythms. These and other circadian rhythms play a role in the pathophysiology of many disease states and may affect the therapeutic efficiency and the side effect profile of prescribed treatments. Hypertension is a prime example of a medical condition whose severity, in terms of blood pressure level, and response to control by medications are impacted by circadian rhythms [**1-3**]. Indeed, a number of common medical conditions exhibit 24-hour patterns in the severity of symptoms or the occurrence of life threatening events. In other words, a person who already has a high level of a particular peptide or protein may experience further aggravation or triggering of an underlying medical condition at the biological time of circulation. Although many medical conditions show specific time patterns, the exacerbation of many medical conditions may not be predictable in time and may be precipitated suddenly without prior symptoms with catastrophic physiological effects.

The peptide hormone angiotensin II plays an important role in blood pressure regulation, both in normotensive and hypertensive individuals. The blood concentration of this peptide fluctuates throughout the day as a circadian rhythm in most persons with peak levels occurring around the commencement of the activity span. The circadian pattern can be different, or even absent, in patients with certain heart diseases such as congestive heart failure. By inducing the activity of mitogenic-activated protein kinases (MAPK's) angiotensin has been shown to stimulate fibroblast production in a dose-dependent manner which in certain cases can eventually lead to atrial fibrillation. [**4**]

Ultimately, it would be beneficial to use polymers that are specifically designed to detect (sense) and capture biologicals such as angiotensin II to correct its over-expression and/or peak concentrations due to circadian patterns in synthesis. Unlike typical pharmaceuticals that are used to react to abnormal physiological conditions or prescribed as sustained-release formulations so as to attain a consistent concentration of medications during the 24-hours, the CIBPs are able to neutralize the biological and chemical triggers of disease and thereby prevent diseases and their symptoms from occurring. For a complete therapeutic effect, the CIBPs will then need to render the over-expressed peptide, whether due to circadian rhythms or a pathophysiologic condition, inactive, and then be cleared from the body.

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2 BACKGROUND

2.1 Molecular Recognition

Molecular recognition is the specific recognition of, and interaction with, one molecule by another [**1**]. This ability of molecules to specifically target another molecule in a consortium of similar molecules is essential to biological and chemical processes. The process typically occurs through non-covalent chemical bonds, including hydrogen bonding or hydrophobic or ionic interactions. Ultimately the molecules are both chemically and spatially complementary. This process can be seen in many living processes, including in the antigen/antibody recognition of the immune system, the binding of an enzyme to a substrate and the interaction of a drug to a biological target. Considered to be natures driving force in life, many scientists have sought to mimic this natural process.

2.2 Natural Recognition Systems

The idea of achieving very specific binding of a target molecule readily echoes many molecular binding events that occur in most biological systems. For example, the evolved immune systems of higher-order organisms exhibit MIP recognition in the complex mechanism of antigen-antibody binding. Antibodies are linear strands of amino acids consisting of two identical polypeptide "heavy

chains" (55 kDa) and two identical polypeptide "light" chains (25 kDa) that are linked by disulfide bridges to form a "Y" structure [**2**]. At the N-terminus of the chains lies the epitope which contain specific chemical functionalities that lead to recognition of a particular portion of an antigen.

It is believed that recognition occurs via ionic or hydrogen complexation between chemical functionalities of antigens and amino acids of the epitope (Figure 2.1). This complexation then triggers the C-terminus of the antibody to bind to phagocytotic cells which consequently engulf and destroy the complex. Many researchers are now attempting to mimic this epitope binding to create artificial antibodies for the treatment of disease [**3**]. Mosbach and collaborators [**4,5**] have considered using these "artificial antibodies" as screening of libraries for potential new drugs, enzyme inhibitors and as targets for protein binding.

In addition to antigen-antibody binding, proteins themselves contain binding domains that contain specific recognition elements for other proteins or biomolecules. Proteins themselves are heteropolymers with specific amino acid sequences. The unique residues of these amino acids dictate the final conformation of the protein, and it is these amino acids acting in unison that form unique binding pockets for biological molecules [**6,7**].

Other research groups have attempted to make completely natural recognition systems by using proteins as the recognition elements. Slade [**8**] lyophilized bovine serum albumin in the presence *p*-hydroxybenzoic acid. Using this technique, binding activity could be enhanced by a factor of approximately 3 over that of the non-imprinted protein. In another approach, Motherwell et al. [**9**] placed chicken albumin into contact with an enzyme mimic of glutathione. By crosslinking the albumin with glutaraldehyde in the presence of the mimic, the researchers were able to memorize the conformational binding of glutathione to the protein. The imprinted protein exhibited an 80-fold increase in enzymatic activity when glutathione was present. The above two protein-imprinting methods not only show that creating natural recognition systems is possible, but that they have the possibility of mimicking complex protein-molecule binding events to create possible artificial enzyme release systems for the treatment of disease.

2.3 Molecular Imprinting

As medical research has expanded and greater understandings of the mechanisms of biological molecules have been discovered, researchers have tried to replicate natural molecular recognition behavior with synthetic molecules and processes. One method to achieve this is by creating synthetic materials that are complementary to biological molecules, both in spatial orientation and chemical functionalities, through a process termed molecular imprinting.

The precise design of macromolecular architecture which is capable of sensing specific molecules has been discussed in the literature for several decades. However it has only been in the recent years that these imprinted systems have emerged in the forefront of biomedical applications. CIBPs have proven their potential as synthetic receptors in several applications. These range from use in liquid chromatography columns to assays and sensor technologies for the detection of small molecules [**10, 11**].

MIP and CIBP technologies involve forming a pre-polymerization complex between the template molecule and functional monomers with specific chemical structures designed to interact with the template, either by covalent chemistry, non-covalent chemistry (self-assembly) or both [**12-16**]. Once the pre-polymerization complex is formed, the polymerization reaction occurs by free radical initiation in the presence of a crosslinking monomer and an appropriate solvent. When the template is removed, the product is a porous matrix with specific recognition elements for the template molecule (Figure 2.2). Thus, CIBP creates stereo-specific three-dimensional binding cavities based on the template molecule of interest [**10, 17, 18**]. The resulting polymer networks can then specifically recognize the template molecule in solution even when in a mixture of other similar molecules.

Although molecular imprinted technology has been reported on for several decades, there are very few systems that describe imprinting with larger molecules such as peptides, proteins and cells. This is due in part to the fact that these molecules are often fluid at various conditions and contain large quantities of functional charge sites. These biological molecules often contain moieties that are both hydrophobic and hydrophilic. It is therefore more difficult to achieve the high recognition selectivity that was reported for some of the original molecular imprinting systems.

2.4 Molecular Recognition Using Biomaterials

For biomedical applications, typically the most successful CIBP systems are ones in which the network is a tightly crosslinked polymeric hydrogel containing acrylic or methacrylic functional monomers and poly(ethylene glycol) (PEG) derivatives. This is due to PEG's biocompatible characteristics. It is known to be able to evade the components of the immune system. By including PEG as a backbone in the hydrogel network, the polymer is able to resist protein and cell adsorption. When charged functional monomers such as methacrylic acid or acrylamide are added into the PEG based system, pockets of recognition can be formed throughout the stealth network, giving way to specific recognitive, biocompatible systems.

Recent studies have shown superior properties of polymer networks that are formed with methacrylates and PEG derivatives [**19**]. Like PEG, methacrylic functional monomers are favored in biomimetic applications because they contain anionic acid groups that can non-covalently complex with template molecules. This allows for binding and unbinding of the template in the polymer network, and echoes natural occurring molecular binding events such as molecular receptor and antibody-antigen recognition. In antigen-antibody binding, it is believed that recognition occurs via ionic or hydrogen complexation between chemical functionalities of antigens and amino acids of the epitope.

2.4.1 Degradable Recognition Systems using Poly(α-hydroxy ester)s as Degradable Components

In trying to create systems that are biocompatible, researchers have also placed a large emphasis on creating degradable systems. This is because the ability to form recognitive degradable networks using a controlled degrading structure has important applications for the treatment of medical conditions, including the use in drug-delivery devices and tissue engineering constructs. These constructs degrade and allow the implant or delivery devices to be easily removed by the body's reticular endothelial system (RES) and avoid the need for extra surgeries for removing foreign body devices. Poly(α-hydroxy ester)s such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA) or combinations of the two (PLGA) have been widely studied and used for such devices. These polymers have ester

linkages that can be hydrolytically cleaved, and that they are known to be extremely biocompatible [**20**].

PLA, PGA, and PLGA are also among the few synthetic polymers that have been approved by the Food and Drug Administration (FDA) for certain human clinical applications. These polymers can be tailored to degrade by designing specific combinations of the PLA and PGA. PGA has an extremely hydrophilic nature and therefore will degrade at a high rate in physiological conditions. PLA, on the other hand, has an extra methyl group which makes it more hydrophobic and causes it to degrade more slowly. Although there has been a lot of emphasis on tailoring specific degradation rates, these polymers undergo heterogeneous degradation as a result of several simultaneous *in vitro* and *in vivo* processes.. Some of these processes include water uptake, swelling, ester hydrolysis, oligomer diffusion, degradation products, and local pH drop [**21-23**].

Diffusion of degradation products out of the polymer structure and into the surrounding medium has been reported to be hindered in larger polymers. This in turn leads to an accumulation of acidic degradation products and a subsequent pH decrease inside the polymer object. The result of the local pH drop inside the polymer leads to autocatalysis and further local degradation. Therefore, although these polymers undergo bulk erosion, there is more rapid degradation in the center than at the edges. Langer and colleagues have reported that when these

polymers are placed in pH 7 phosphate buffered solution (PBS) at 37°C and allowed to degrade, the acid buildup in the center of the polymers can lead to a local pH drop below pH of 3 [**21**].

2.5 Applications of Molecular Recognition Systems

The field of molecular imprinting has flourished in the past two decades, and several systems have been used successfully in practice. In particular, molecularly imprinted polymers show a lot of promise in chromatography and separation columns [**10**]. However, recently the field has expanded to include potential drug-delivery and pharmaceutical applications.

Of recent note DDS are the efforts by our group and others to use environmentally responsive, MIP hydrogels as carriers for controlled release of therapeutic compounds. Hydrogels have been used as prime carriers for pharmaceutical applications, predominantly as carriers for delivery of drugs, peptides or proteins. They have been used to regulate drug release in reservoir-based, controlled-release systems or as carriers in swellable and swelling controlled-release devices [**24-26**]. Hydrogels can be rendered sensitive to physiological conditions due to the presence of specific functional groups along their backbone polymer chains. The swelling behavior and associated release kinetics of these gels may be dependent of pH, temperature, ionic strength, or

even drug concentration [**25**]. The technology has evolved to achieve sustained delivery of large molecules, such as high molecular weight peptides for long periods of time (e.g., days and months), and certain groups have recently used MIP polymers to administer the sustained controlled release of small-molecular weight drugs for therapeutic purposes. For example, PEG hydrogels as contact lenses have been composed with imprinted sites for the drug timolol [**27**]. The interaction of the imprinted sites with timolol must be overcome by the uptake of water and this slows the release of this drug into the eye. Thus, MIP contact lenses exhibited longer release times than non-imprinted polymers loaded with the drug [**27-28**].

Unfortunately, current DDS are often limited because they cannot respond in a real time mode to the biological needs of a patient. Every person's physiology and pathological conditions vary slightly. Medical conditions also vary through the course of time, and many disease states often possess unpredictable episodes or unnoticeable symptoms that may lead to catastrophic physiological effects. Therefore, it would be advantageous of future DDS to function on a real time basis so as to be able to accurately sense and determine changes in significant biological markers and then actively respond by releasing a therapeutic agent or a sensible signal. Ideally these systems should also be able to self regulate and effectively be able to switch on and off depending on the

therapeutic need. Some of the recent developments in actively responding systems are described below.

Tanaka and collaborators [**28-32**] have described one recognition system in which polymer gels recognize molecules in a manner very similar to the process in which proteins recognize them. In other words, recognition capabilities are based on a specific molecular conformation, and these gels are able to change in and out of a particular conformation based upon environmental cues, such as ionic strength, temperature, or pH. These gels are able to memorize certain configurations based on spatial assemblies of groups of their monomers. Essentially, this group creates a thermo-sensitive polymer, and then polymerizes previously end-shielded post-crosslinking monomers in the presence of a template or target molecules (negatively charged molecules that interact with positively charged functional monomers), and the resulting gel is a MIP network with thermo-responsive properties.

When Tanaka et al. compared the recognition aspects of the gel, it was shown that the presence of the template during the additional post-crosslinking step resulted in 3-5 times higher affinity compared to random post-crosslinking. They were also able to show that the functional monomers with carboxyl groups formed complexes with divalent ions when polymerized, but upon swelling the affinity for the divalent ions disappeared. However, when the temperature was

increased the gels would shrink, the affinity was recovered, and the original relative position of the carboxyl groups were recalled in effect memorizing the original polymer formation. Non-imprinted gels had decreased affinity for divalent ions and trouble forming pairs due to polymer frustrations.

Although these MIP systems show multiple-point absorption, they have yet to recognize molecules other than charged species. However, it is interesting to note that the presence of acidic groups is a common feature in drug substances including antibiotics, glucocorticoids and cholinergic drugs [**33**]. Future uses of these systems show great promise in the drug-delivery field due to the reversibility of drug release and re-uptake as a function of temperature. They could be applied to pathological conditions which involve local changes in temperature or by triggering the on/off release of drug due to externally applied local hyperthermia.

Perhaps a more specific active recognition/release process is one using a competitive binding system. This system could respond to the individual patient's therapeutic requirements and deliver a specific amount of drug in response to a biological stimulus [**34**]. An example of this system is a reversible antigen responsive hydrogel [**35**]. This gel uses the reversible binding between an antigen and an antibody as the crosslinking mechanism in semi-interpenetrating networks (semi-IPNs). Miyata et al. [**35**] synthesized a semi-IPN which consisted

of a polymer containing rabbit antibody (IgG) and goat anti-rabbit as the antigen. Upon placing free antigen, rabbit IgG, into a solution with the IPN, the hydrogel swelled. This is due to the competition between free and polymer-immobilized antigen. The group was also able to show that the hydrogel swelling was reversible by removing the free antigen, and that this relationship was based on free antigen concentration. In order to demonstrate the relevance this system has in a DDS, drug was entrapped in the network and shown to release proportional to stepwise changes in antigen concentration. While not an MIP system, this network shows once again that hydrogel systems could be tailored to release a drug in response to an external stimulus.

The competitive binding method outlined above can be extended to creating networks that include non-imprinted drugs that are released when the template molecule appears in the surrounding medium. For this application, the MIP network must possess a weak affinity for a therapeutic drug and a strong affinity for the template molecule. When in the presence of the imprint molecule, the network binds this molecule, which consequently leads to the release of drug. Such an MIP system has been shown to release hydrocortisone upon recognition of testosterone [**33**]. Other MIP competitive binding systems include the use of anesthetic drugs, methylzantines and/or other sterols to trigger the responsive release of drug [**33**].

There are several physiological and pathological conditions that release particular analytes as direct indicators of the progression of the disease state. Therefore, a need exists to develop systems that are environmentally responsive hydrogels for given analytes. These analyte sensitive systems could be molecularly recognitive for biologicals produced at certain intervals and be able to release therapeutic agents in response. One such system is a calcium–responsive bioerodible DDS devised by Goldbart and Kost [**36**]. This system is a starch-cellulose matrix that contains α-amylase, which is a biological protein regulated by calcium. As calcium enters the hydrogel system, bound α-amylase binds with this ion and is cleaved to become active. This active calcium can then be utilized by the body, and these gels can eventually be used to treat calcium-dependent diseases such as osteoporosis.

In future technologies, imprinted gels or chains possessing certain macromolecular architecture with binding abilities could be used as the sensing elements within analyte-sensitive controlled-release systems. The design and implementation of imprinted recognition release systems would be challenging, but certainly one can envision imprinted gel films loaded with therapeutics. It could be reasoned that binding of target molecules to active sites or specific chemical groups could change the overall ionic character or hydrophilicity or hydrophobicity and in turn cause the swelling of a polymer gel for release of a drug. Kataoka and coworkers [**37,38**] have shown this effect using a hydrogel

that contains a borate-glucose complex. These gels exhibit a sharp transition in degree of swelling with respect to glucose concentration. As glucose binds, the network becomes more hydrophilic due to the formation of a charged template-binding group complex. Thus, as the authors speculate, this type of polymer gel can be used as a chemical valve to regulate drug release in response to glucose concentration, for use as a DDS in the treatment of diabetes.

A particularly promising idea is the use of imprinted systems in medical applications that impose the principles of chronobiology. Recently, there has been much attention given to chronopharmacological DDS. These DDS are designed to synchronize the delivery of therapeutic agents to biological rhythm (24-hour and other periods)-driven requirements. These systems are very important especially in the area of endocrinology and in delivery of vaccines. For example, it has been shown that the treatment of hypopituitary dwarfism by administration of human growth hormone releasing hormone (GHRH) is more effective when the GHRH is administered in a pulsatile manner [**39**].

It is a common practice in clinical pharmacology to assume the pharmacokinetics and pharmacodynamics of medications are not influenced by the time of day (with reference to circadian rhythms) of their administration. However, there is convincing evidence that shows this assumption is not always valid. It is now

well established that nearly all functions of the body, including those influencing pharmacokinetic parameters, display significant circadian variation [**40,41**].

These new findings have lead to the development of a new research area, that of chronopharmacology, which is a study of coordinating biological rhythms (chronobiology) with medical treatments. Chronobiology is the study of the body's biological rhythms and time structure. All living creatures evolved in a setting that is characterized by predictable changes in time, e.g., cyclic relationships of the sun, earth and moon. Early life-forms were strongly helio-dependent. Living organisms had to store energy during periods of daylight for use during periods of darkness. Adaptability to the influence of the circadian patterns of our planet was a condition of life, and it is apparent that all organisms have incorporated and retained vital circadian and other periodicities in their genetic code.

Many chronic and acute disorders have a prominent circadian pattern of symptom appearance and severity. For example, (i) allergic rhinitis is an early morning disorder, (ii) patients with epilepsy have seizures during the day only, or the night only, or some have a more random pattern. (iii) peptic ulcer disease is worse late at night and early morning hours when the acid secretion of the stomach is the highest [**42**], (iv) asthma attacks typically occur during the night [**43-45**], (v) thrombotic and hemorrhagic stroke risk are greatest in the morning

while (vi) cholesterol synthesis is greater in the late evening [**46**]. Several cardiovascular diseases show circadian patterns, such as myocardial infarction, stroke and angina pectoris [**47, 48**].

The future applications of molecular imprinting could have major implications for chronotherapy. By introducing MIP polymers into the bloodstream, it is possible for them to preferentially bind to over-expressed undesirable biologicals when they are in their elevated state and ultimately reduce them to safe plasma levels before any negative physiological effects occur (Figure 2.3).

2.5.1 Angiotensin II and the Renin-Angiotensin-Aldosterone System

Novel imprinted chronotherapeutic systems would be particularly promising when trying to regulate over-expressed peptides or proteins, whether at particular times of the day and night and/or throughout the 24-hours. Angiotensin II is an example of peptide that is often overexpressed and could benefit from such a therapeutic system.

The renin-angiotensin-aldosterone system (RAS) is a well defined complex regulatory system with many identifiable actions. However it is best known for being a regulatory system for the conservation of salt and blood volume, and the preservation of blood pressure. The major end product of the RAS cascade is

angiotensin II. Angiotensin II is the active form of angiotensin I. Angiotensin I is first cleaved by an angiotensin converting enzyme (ACE) which creates angiotensin II. Angiotensin II then acts on either type 1 or type 2 angiotensin receptors.

Angiotensin II is an octapeptide with the following structure: asp-arg-val-tyr-ile-his-pro-phe. It is naturally cleaved by three aminopeptidases in the body which are call aminopeptidase A, B, and C. Aminopeptidase C cleaves the angiotensin II between the seventh and eighth amino acid to create angiotensin (1-7). It is this form of angiotensin that counteracts the effects of the angiotensin II. The most common way to treat a patient who displays an over-expression of angiotensin II is to administer ACE inhibitors or angiotensin receptor blockers (ARBs). However, both these treatments can exert side effects, including birth defects, and ACEs, in particular, are known to elevate potassium levels and to induce cough and dizziness. Extreme side effects include kidney failure and decrease of white blood cells [**49**].

2.6 Figures

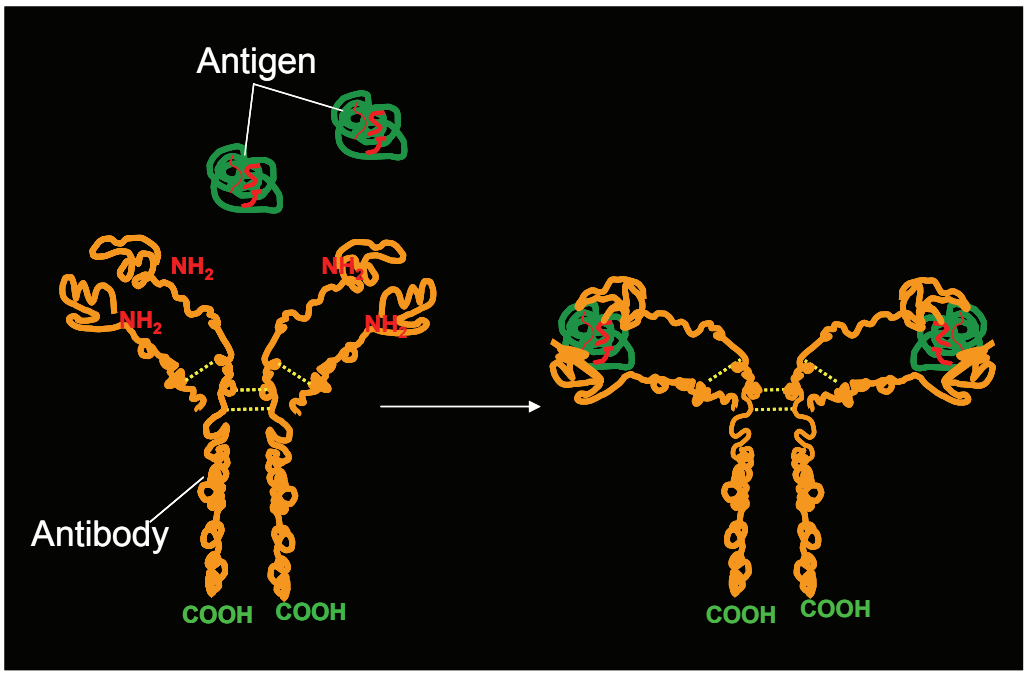

Figure 2.1: Antigen/Antibody Molecular Recognition
Antibodies are "Y" structures that recognize antigens via a very specific arrangement of amino acids. [**18**]

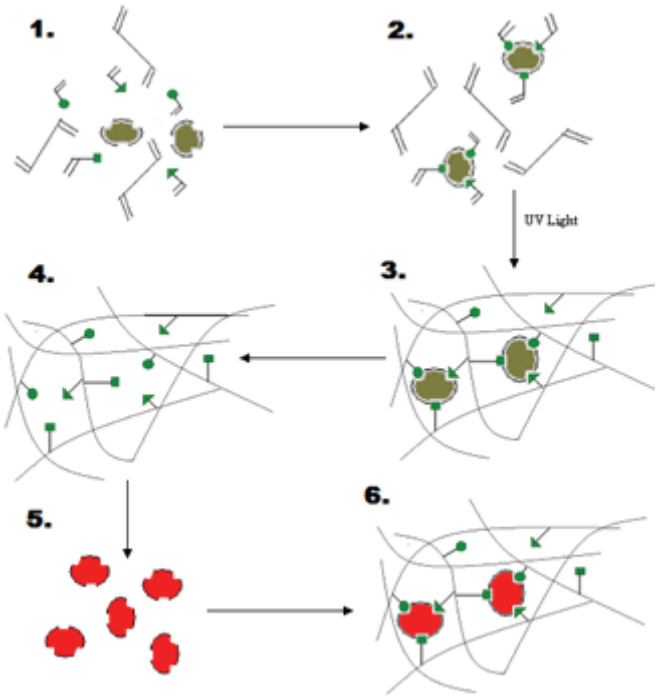

Figure 2.2: (1) Template molecules are mixed with free functional monomers and crosslinking agent. (2) Functional monomers bind to reactive sites on the surface of the template molecule. (3) The monomers react with the crosslinker to form a crosslinked template through UV photopolymerization. (4) Molecules are washed from the template leaving a recognitive scaffold. (5,6) New molecules occupy spaces formerly held by the template molecules. [**18**]

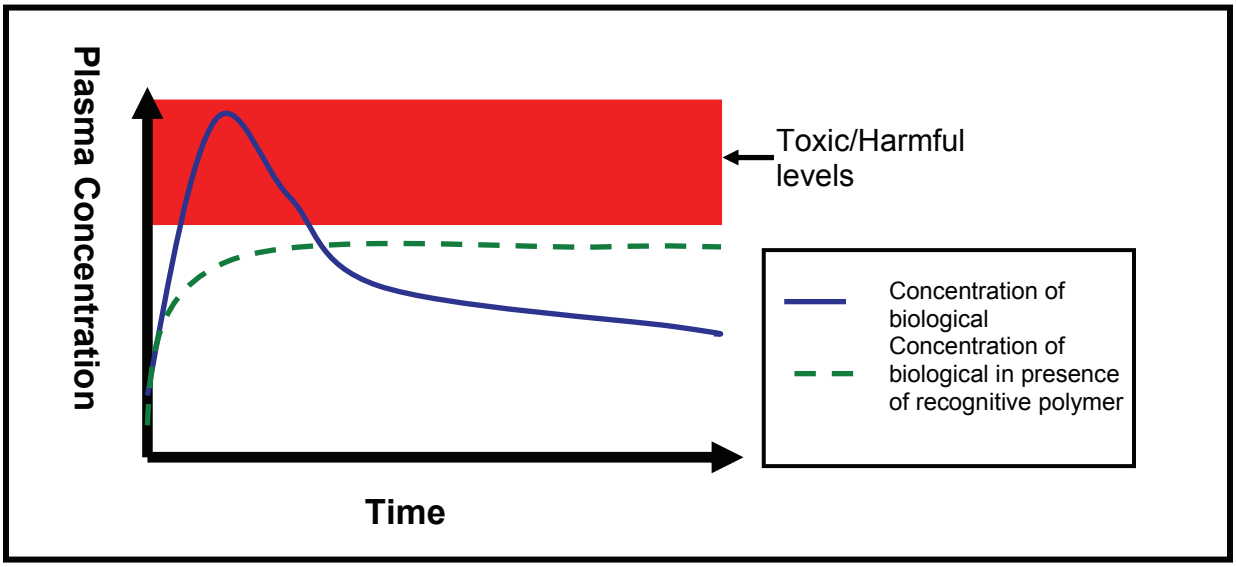

Figure 2.3: Schematic showing that the presence of imprinted microspheres in the blood are able to absorb undesired biologicals and reduce their concentration in the plasma to safe levels.

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3 OBJECTIVES

The objective of the work here has been derived from the main hypothesis which is that tailor-made biodegradable CIBPs can recognize angiotensin II using acrylamide and related comonomers. The ensuing materials can act as biologically responsive carriers for drug delivery. Thus, a new generation of intelligent, responsive drug delivery systems will be created.

The specific aims of this research are as follows:

(1) To fabricate, optimize and characterize nondegradable polymer systems prepared from configurationally imprinted biomimetic polymers which specifically recognize angiotensin II.

(2) To synthesize and characterize a hydrolytically cleavable poly(α-hydroxy ester) crosslinking agent (crosslinker).

(3) To fabricate degradable configurationally imprinted biomimetic polymers and characterize their physicochemical effects and chemical interactions with the template peptide angiotensin II.

(4) To conduct an *in vitro* characterization of the configurationally imprinted biomimetic polymers, measuring the biocompatibility of the constructs and the cytotoxicity of the degradation products.

4 FABRICATION OF NONDEGRADABLE CONFIGURATIONAL BIOMIMETIC IMPRINTED POLYMERS SELECTIVE FOR ANGIOTENSIN II

4.1 Introduction

By explicitly choosing components and tailoring reaction conditions it is possible to effectively design a polymer network that is specifically imprinted for an undesirable biological. These polymer networks will then be able to rapidly recognize the biological molecules in physiological conditions.

When designing experimental conditions, several factors need to be considered. Specifically it is important to examine template interactions with functional monomers, percentage of crosslinker, and the solvent conditions in which the reaction will be run. Choosing the functional monomers that will be used is based on the template's size and charges.

In configurational biomimetic imprinting, recognition occurs through specifically localized hydrogen bond complexes. It is therefore desirable to choose monomers that can provide opposite hydrogen bonding functionalities to the template molecule. Since these systems will ultimately be placed in physiological conditions, it is also important to mimic natural functionalities of amino acids and ensure biocompatibility. The next factor that needs to be considered is choosing the appropriate amount of crosslinker for the mesh network. The recognition is

based upon specifically sized binding cavities. If an insufficient amount of crosslinker is used, the binding cavities will swell and distort in solution. However, if too much crosslinker is used, the network that will form will be so dense that the template would be unable to move into and out of the system. Finally a solvent needs to be chosen in which all components will be mutually soluble and for which the template will have a similar hydrodynamic radius to what it has in physiological binding conditions.

With the above considerations in mind, the components of the system that specifically recognizes angiotensin II were chosen to be poly(ethylene glycol) monomethacrylate and acrylamide as the functional monomers, poly(ethylene glycol) dimethacrylate as the crosslinking agent and dimethyl sulfoxide (DMSO) as the solvent (Figure 4.1).

4.2 Materials and Methods

4.2.1 Fabrication of Angiotensin II Imprinted Polymers

Imprinted polymer networks were prepared by fast, UV-initiated, free radical polymerization reactions of acrylamide and poly(ethylene glycol) monomethacrylate 400 (PEGMA 400) as the functional monomers, poly(ethylene glycol) 1000 dimethacrylate (PEGDMA 1000) as a comonomer and the crosslinking agent, and angiotensin II as the template molecule.

In a typical synthesis of an imprinted network, 0.095 g of (PEGMA 400) (Polysciences Warrington, PA.), and 0.13 g of acrylamide (AAm) (Sigma-Aldrich, St. Louis Missouri), were placed in an amber bottle and dissolved in 0.6707 g DMSO (Fisher Scientific). The monomers are in a 1:1 molar feed ratio of ethylene glycol units:AAm. Then 0.027 g of angiotensin II was added. The solution was allowed to sit for 20 minutes in order to form prepolymerization complexes. Then 0.2255 g of (PEGDMA 1000) crosslinker was added. After the monomer solution was mixed and allowed to go into solution, the photoinitiator 1-hydroxyclyclohexyl phenyl ketone (Irgacure 184®) (CIBA-GEIGY Hawthorne, NY) was added.

The amber bottle was then covered with parafilm and placed in a nitrogen atmosphere. A needle was placed through the parafilm and nitrogen was allowed to bubble through the solution for 20 minutes in order to remove the oxygen, a free-radical scavenger. The solution was then pipetted between two glass plates which were separated by a 700 μm Teflon spacer. The glass plates were then exposed to UV light (Dymax 2000_EC Light Curing System, Torrington, CT) for 20 minutes at an intensity of 16 mW/cm^2. The resulting polymer was then removed from the glass plates and washed in a 300ml solution of acetonitrile with 5% acetic acid for 7 days. This was to ensure template extraction and the removal of any unreacted monomer. The polymer was then dried in a vacuum oven at 35°C for 2 days. It could then be cut into 12 mm diameter disks or

crushed into particles ranging in size from 75-150 μm. Control polymers were synthesized in the same manner with the exception that no angiotensin II was added.

In order to optimize the recognition of the polymer for angiotensin II the previously described synthesis was performed several times, varying first the molar ratio of template to functional monomer and then varying the crosslinker mole percentages in the formulations. The various ratios of template to functional monomer were 1:8, 1:16 and 1:32. After determining the best template to monomer ratio, the crosslinker percentages were varied. The various formulations that were produced included networks with 10 mole%, 20 mole%, 40 mole% and 80 mole% PEGDMA 1000.

The polymer formulation with the most specific recognition was then used as a template for adding a third functional monomer in order to increase recognition. The third monomer that was added was methacrylic acid (MAA). The networks were fabricated as previously described with the exception that MAA was added in a 1:1:1 molar feed of EGMA:AAm:MAA.

4.2.2 Template Removal Study

In order for selective recognition/binding to be successful, it is important to remove the original template from the binding cavity. Studies of the wash solutions were therefore conducted to determine that the template had been removed from the polymer. After each wash, three 200 µl samples of solution were placed in a UV/Vis 96-well plate. This plate was then read by a UV/Vis microplate reader at a wavelength of 215 nm. The results were then compared to a standard curve of angiotensin II concentrations. They were then subtracted from the initial amount of angiotensin II added during synthesis.

4.2.3 Swelling Studies

The imprinted networks were then evaluated by performing dynamic swelling studies. Briefly the swelling studies were performed by placing dry polymer disks (12 mm diameter) in a solution of pH 7.4 PBS at 37°C. Ionic strength was controlled with sodium chloride. The disks were removed, blotted dry and weighed at regular intervals for 20 minutes. The weight swelling ratio, q, was calculated as the swollen weight/dry weight of the polymer disk. The time allowed for swelling was calculated by using an approximation of in vivo swelling as shown below:

$$t = \frac{l^2}{D} = \frac{(0.07cm)^2}{10^{-6}\ cm^2/s} \approx 11\min \qquad (4.1)$$

where t is time, l is the thickness of the of the film, and D is the diffusion coefficient of water into the dry film.

4.2.4 Specific Recognition Studies

To determine if angiotensin II was successfully imprinted into the polymer networks, recognition studies were performed. The various polymer formulations were placed in the presence of angiotensin II or SVA angiotensin. SVA angiotensin is a synthesized derivative of angiotensin II in which three amino acid moieties were substituted. Its structure is **ser**-arg-val-tyr-**val**-his-pro-**ala.** It has a molecular weight of 958 and a charge at pH 7 of +1. Both of these characteristics are similar to angiotensin II. The binding studies were also conducted in both DMSO, the solvent used during the synthesis, and phosphate buffered solution (PBS) with a pH of 7.4. The latter was used to simulate physiological conditions.

In a typical experiment, dry imprinted polymers were cut into 12 mm diameter disks and weighed. A disk was then placed into a 15 ml conical tube which had either solutions of 1mg/ml angiotensin II in DMSO, 1 mg/ml angiotensin II in PBS, 1mg/ml SVA angiotensin in DMSO or 1 mg/ml SVA angiotensin in PBS. Nano imprinted control polymers of the same formulation were also cut into 12 mm disks, and placed into their own 15 ml conical tubes containing the solutions described above. The tubes were then placed on a rotating mixer set at 25 rpm. At time points of 0, 1, 2, and 3 hours, three 200 µl samples of supernatant were

removed and placed into a UV/Vis 96-well plate. The plates were then read by a UV/Vis microplate reader at a wavelength of 215 nm. The results were then compared to standard curves of known concentration of both angiotensin II and SVA angiotensin. The peptide concentration in the supernatant was determined from this. This concentration was then subtracted from the original solution concentration. The final result was then divided by the weight of the original dry polymer to determine mg bound/mg polymer. All experiments were repeated in triplicate for each formulation of the polymer.

The best polymer formulation was then tested for specific recognition against a physiologically relevant peptide. The binding studies were performed as previously described; however, instead of using SVA angiotensin, another synthesized peptide which is present in the blood stream, antidiuretic hormone (ADH), was used. ADH is a biologically relevant peptide which is of similar size to angiotensin II. It consists of the nine amino acids: cys-tyr-phe-gln-asn-cys-pro-arg-gly.

4.3 Results and Discussion

4.3.1 Template Removal Studies

For a recognitive system to be successful in recognizing its template molecule, it is important to ensure that the template used during the polymerization process

is completely removed from the system. This ensures that the binding cavities of the imprinted networks are free to interact with the templates placed in the binding solutions. The polymer networks were therefore examined in order to determine that the original template molecules were completely removed from the system.

The results of the template removal study can be seen in Figure 4.2. In all cases approximately 87% of the original template was removed. There are several possibilities to explain the remaining 13%. One explanation would be that it is permanently entrapped in the polymer. It is also possible that due to polymer/template thermodynamic interactions, the template was not 100% soluble in the prepolymerization solution [**1**]. If the template was not completely dissolved, it is possible that it was not actually incorporated into the network during the polymerization process. Finally, it is possible that a small quantity of the template is located deep within the polymer network and takes more time to diffuse out. If it leaves the polymer in extremely small quantities, it may be outside the sensitivity range of the UV/Vis and therefore undetectable.

4.3.2 Specific Recognition Studies

The goal of the design of the imprinted network was to create a system that was sensitive in recognizing its template molecule when compared to the sensitivity of the nonimprinted control network, and to create a system that was selective for

that template over competitive molecules. To assess the success of such an imprinted network, recognition studies were performed. To achieve the most recognitive network, molar ratios of the template to functional monomers and the molar crosslinking percentages were varied and investigated. The most sensitive network was then tested for its selectivity over competitive peptides.

The calculations for recognition were based on the amount of equilibrium peptide recognition, P_{rec} (units of mg-peptide/g-polymer), in the polymers. It was calculated by a simple mass balance:

$$P_{rec} = \frac{V(C_{initial} - C_{equilibrium})}{M_p} \qquad (4.2)$$

where $C_{initial}$ is the starting peptide concentration, $C_{equilibrium}$ the final concentration after of peptide in supernatant after loading, V the volume of peptide solution, and M_p the mass of polymer disks used. The percentage of recognition of MIP to control was then calculated. In our case the original concentration of peptide in solution was kept at 15 mg/ml and the polymer disks weighed approximately 0.368 g. Therefore the maximum possible amount of mg peptide bound to grams of polymer was determined to be 40.76. This was considered to be 100% binding. The results are therefore reported as a percentage of this binding.

In the first test of recognition, the imprinted networks were investigated for sensitivity against their nonimprinted controls by varying their molar template to functional monomer ratio. The goal of this experiment was to determine the optimal ratio of free monomer functionality to the complimentary template functionality. An excess of free monomer functionality can lead to the polymer being charged which can cause nonspecific absorption of molecules. On the other hand, if there is not a high enough ratio of monomer functionality to the complimentary template functionality, the recognition cavities in the polymer will not have enough specific sites to selectively recognize the template.

The recognitive network used in this study was poly(ethylene glycol-g-acrylamide) with a 20 mol% crosslinking density. The molar ratios of template to functional monomer were varied to be 1:8, 1:16 and 1:32. These values were chosen based on typical literature values [**2**]. The results of this recognition study can be seen in Figure 4.3. The 1:8 recognitive network was determined to be approximately 22% more sensitive than its nonimprinted control. The 1:16 and 1:32 were determined to be 20% and 8% more sensitive, respectively. Therefore the 1:8 molar ratio was chosen to be the optimal ratio for this network.

Another important aspect in optimizing the recognition of the networks is determining the optimal crosslinking percentage. This is due to the fact that the recognitive networks are hydrogels and have the propensity to swell. This

swelling is partially based upon how loosely crosslinked the systems are. If the networks are too loosely crosslinked they will exhibit a large amount of swelling and could ultimately affect the stereo-specific three dimensional space of the recognitive cavity. Without correctly positioned monomer functionality in the recognitive cavity, the imprinted network will loose the ability to be sensitive for its template. On the other hand, if the network is too tightly crosslinked, molecules, including the template molecule, will experience difficulty moving through the network. This will ultimately reduce the time in which the system can detect the imprinted molecule and could possibly reduce the total amount of template detected.

With the above considerations in mind, the poly(ethylene glycol-g-acrylamide) network with a 1:8 template to functional monomer molar ratio was varied to contain crosslinking percentages of 10 mol%, 20 mol%, 40 mol% and 80 mol%. The results can be seen in Figure 4.4. The recognitive network exhibited 17% sensitivity to its nonimprinted control when it contained 10 mol% crosslinker. It exhibited a sensitivity of 21% for the 20 mol%, 25% for the 40 mol% and approximately 14% for the 80 mol%.

In order to further increase the recognition capabilities of the polymer network, a third functional monomer was included. Methacrylic acid was added to the network to add potential ionic bonding sites which ultimately provides more

complementary function to the template molecule angiotensin II. Recognition studies showed that the addition of the third functional monomer did slightly increase the recognition of the network to approximately 28% (Figure 4.5). This could be in part due to the fact that methacrylic acid, the third functional monomer, provides ionic bonding sites. Purely covalent bonding, such as hydrogen bonding, is not always the most efficient way to recognize, because there is often competition between the solvent and the template. Therefore by introducing non-covalent bonding sites, it is possible to increase recognition [**3**].

Finally, it was the goal of this work to not only create a sensitive recognitive system, but also a highly selective one. Therefore, the most recognitive formulation of pegylated poly(acrylamide-co methacrylic acid) with a template to functional monomer molar ratio of 1:8 and a crosslinking percentage of 40 mol% was tested for its selectivity by performing recognitive studies with competitive peptides (Figure 4.6). The polymer was 27% more selective for angiotensin II than the synthetic SVA angiotensin. However when the polymer was compared to the physiologically available ADH, the selectivity was increased to 32%.

The aforementioned recognition studies were conducted in DMSO, the solvent used during the synthesis process. However, with the ultimate goal being to develop systems that can be used in physiological environments, the recognitive polymers were also tested for their selectivity and sensitivity in a physiologically

relevant solvent, PBS (Figure 4.7). Although recognition in PBS is decreased compared to DMSO, there is still selective binding in the imprinted systems for Angiotensin II over SVA angiotensin and ADH. The recognition study shows the imprinted network was 6% more selective for angiotensin II than SVA angiotensin and 7% more selective than ADH.

In recent years the field of molecular imprinting has gained acceptance as a major scientific endeavor, and there has been a dramatic increase in the number of journal articles published. With the promise of being able to use these systems in biotechnology and pharmaceutical applications, various groups have begun using several methods to try to imprint larger molecules such as peptides and proteins. These have included processes such as radiation-induced graft polymerization [**4**], dry phase separation [**5**] and surface imprinting [**6**]. Unfortunately, there is still no standard in the field that enables uniform comparison between imprinted systems. Ultimately, recognitive polymers can be classified by affinity, selectivity and capacity. However, even with the various imprinting techniques, very few imprinted systems have produced the results that match the association of a protein or enzyme for a particular ligand [**7**]. The most successful imprinted systems have been for small molecules. Mosbach and collaborators have reported up to a 99% selectivity for theophylline over caffeine [**8**]. Imprinted networks for proteins and peptides, however, have been met with

much less success. A 30% increase in selectivity is still considered on the high end. Therefore, the results presented in this study are promising.

4.4 Conclusions

Configurationally imprinted biomimetic recognitive networks with specific recognition for the oligopeptide angiotensin II were fabricated. The networks showed 28% sensitivity over their nonimprinted controls, and up to 32% selectivity over competitive peptides.

Although much research has been done investigating MIPs using small molecules as the templates, less emphasis has been placed on larger, more complex templates, such as peptides and proteins due to such factors as inherent diffusion limitations of larger molecules and the sensitive structure/function relationship of peptides and proteins. Therefore, the results of this work are very significant because it shows that it is possible to create a recognitive network for an oligopeptide. The selectivity of this network is also relatively higher than most literature reports for templates of similar size.

4.5 Figures

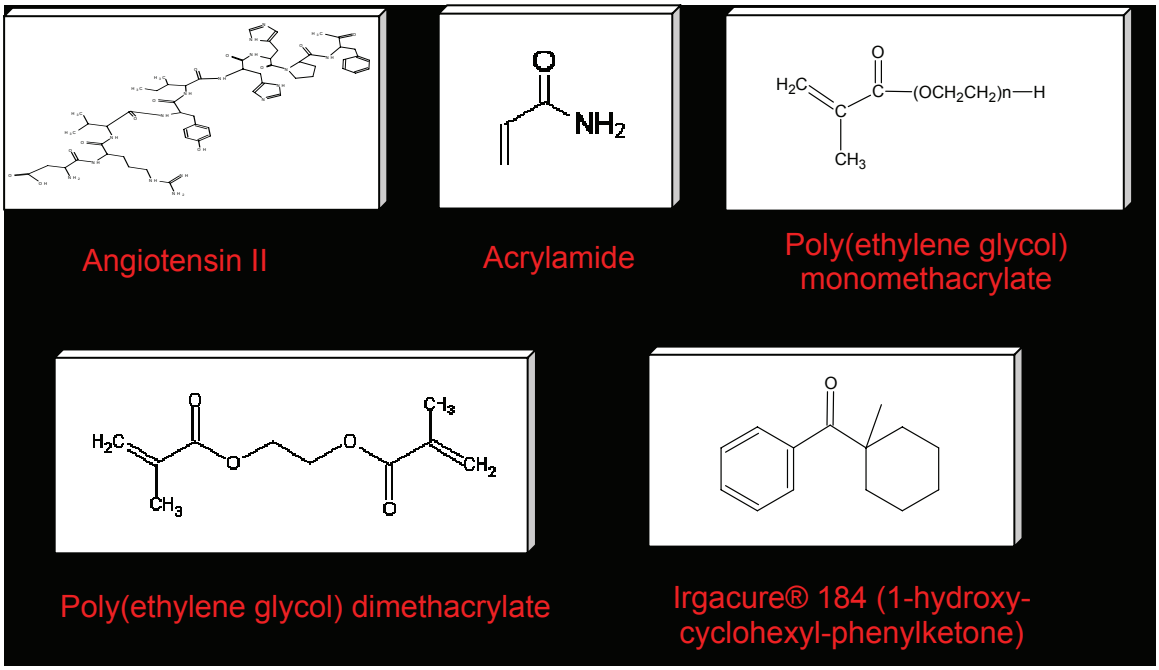

Figure 4.1: System Components of Recognitive Networks

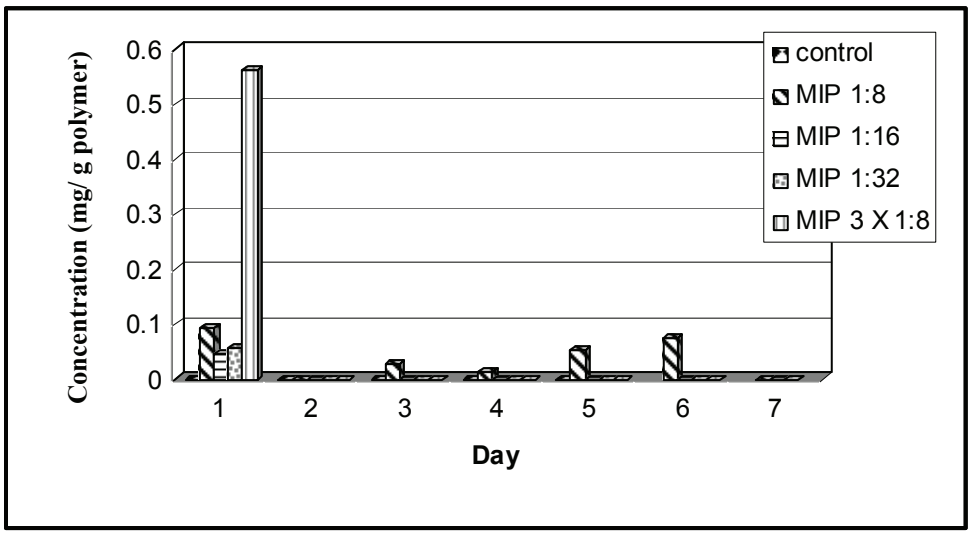

Figure 4.2: Template Removal Study
The polymer was washed for 7 days in acetonitrile with 5% acetic acid. A sample was taken from each wash, and measured by UV spectroscopy at 215nm to determine concentration. The Figure shows ~87% of Template has been removed in each case.

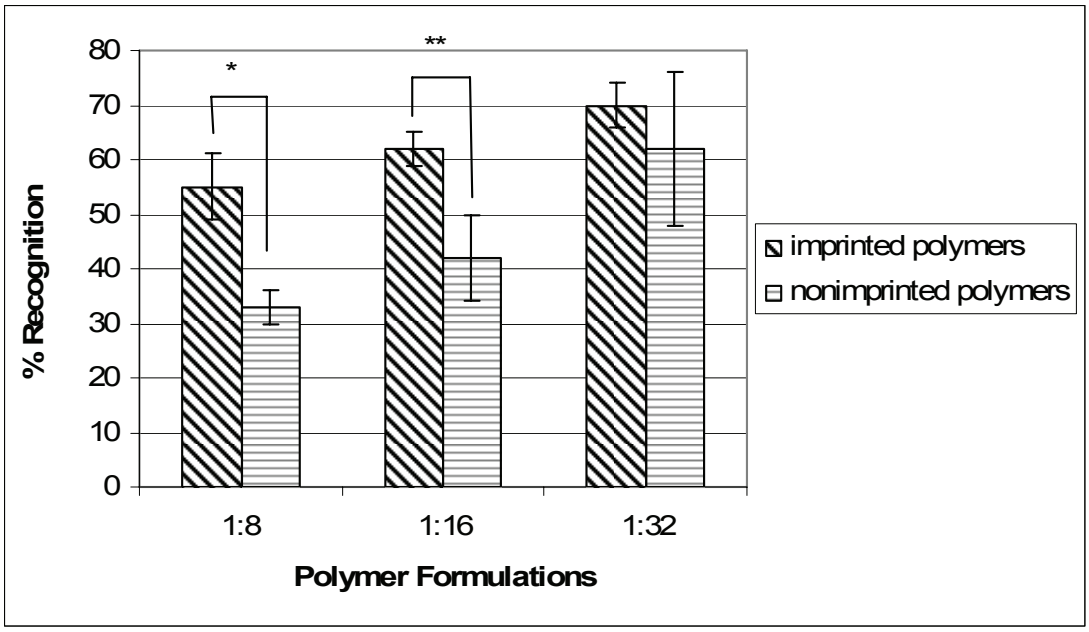

Figure 4.3: Recognition Studies with Varying Template to Functional Monomer Molar Ration

Recognition studies performed with poly(ethylene glycol-g-acrylamide) networks with a 20 mol% crosslinking percentage. Template to functional monomer molar ratios were varied between 1:8, 1:16 and 1:32 Figure shows percent of angiotensin II that is bound to the recognitive polymer at 3 h. n=3 * p=0.0024 ** p=0.01

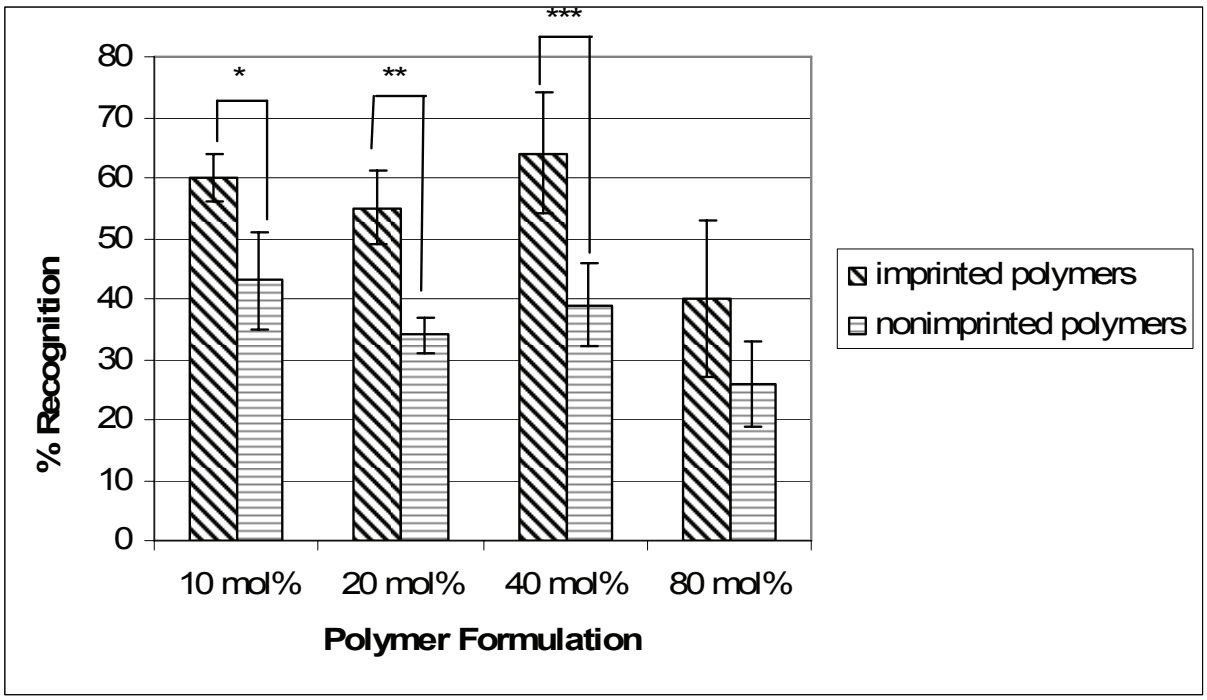

Figure 4.4: Recognition Studies with Varying Crosslinking Percentage
Recognition studies performed with poly(ethylene glycol-g-acrylamide) networks with a 1:8 template to functional monomer molar ratio. Crosslinking percentages were varied from 10 mol% to 80 mol% Figure shows percent of angiotensin II that is bound to the recognitive polymer at 3 h. n=3 * p=0.015, ** p=0.003, *** p=0.012

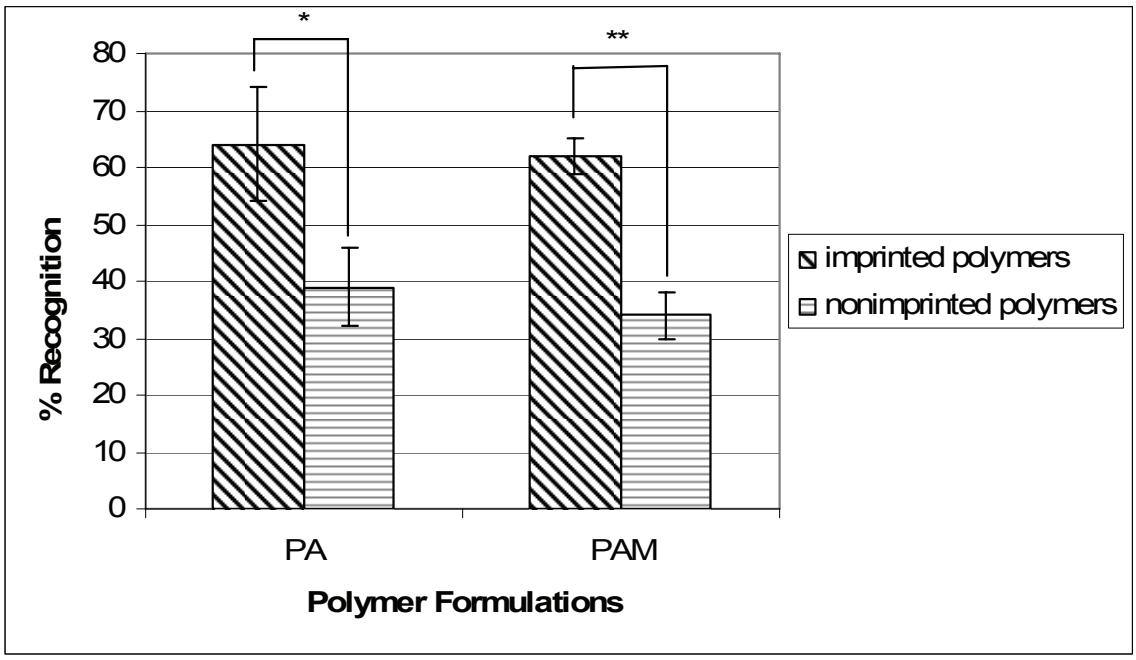

Figure 4.5: Recognition Studies with Various Polymer Formulations
Recognition studies performed with poly(ethylene glycol-g-acrylamide) networks (PA) or pegylated poly(acrylamide-co-methacrylic acid) (PAM) networks, each with a 1:8 template to functional monomer molar ratio and a crosslinking percentage of 40 mol%. Figure shows percent of angiotensin II that is bound to the recognitive polymer at 3 h. n=3 * p=0.012, ** p= 0.0003

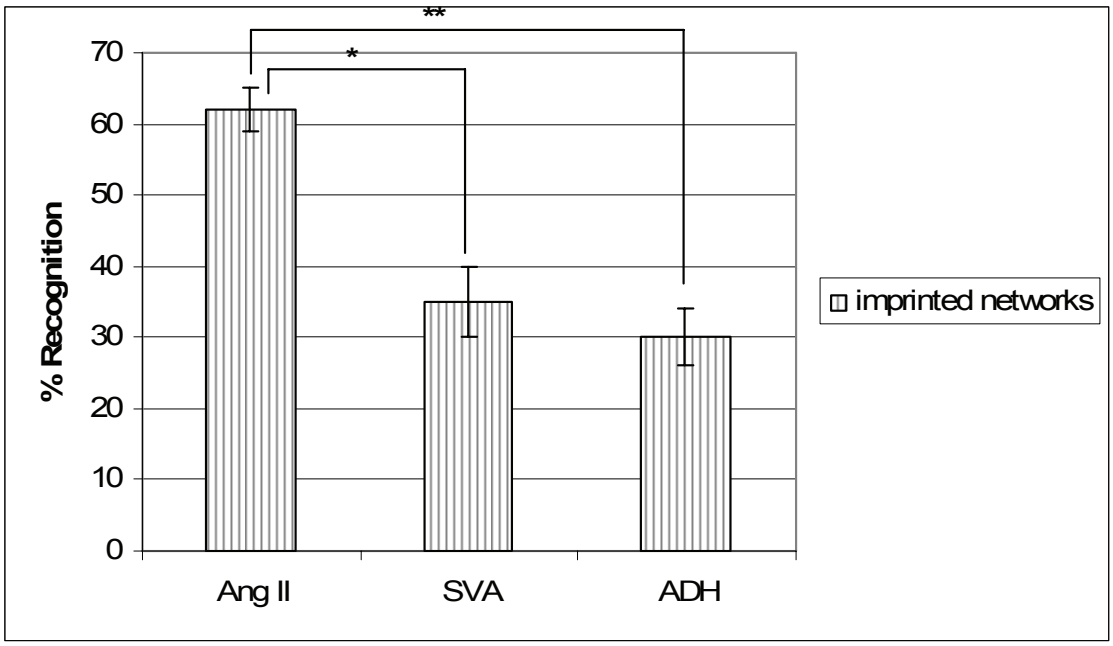

Figure 4.6: Specific Recognition of Imprinted Polymer
Recognition studies performed with pegylated poly(acrylamide-co-methacrylic acid) (PAM) networks with a 1:8 template to functional monomer molar ratio and a crosslinking percentage of 40 mol%. Imprinted polymer was placed in a 1 mg/ml solution of either angiotensin II (AngII), SVA angiotensin (SVA) or ADH. Figure shows percent of peptide that is bound to the recognitive polymer at 3 h. n=3 * p= 0.0007, ** p=0.0002

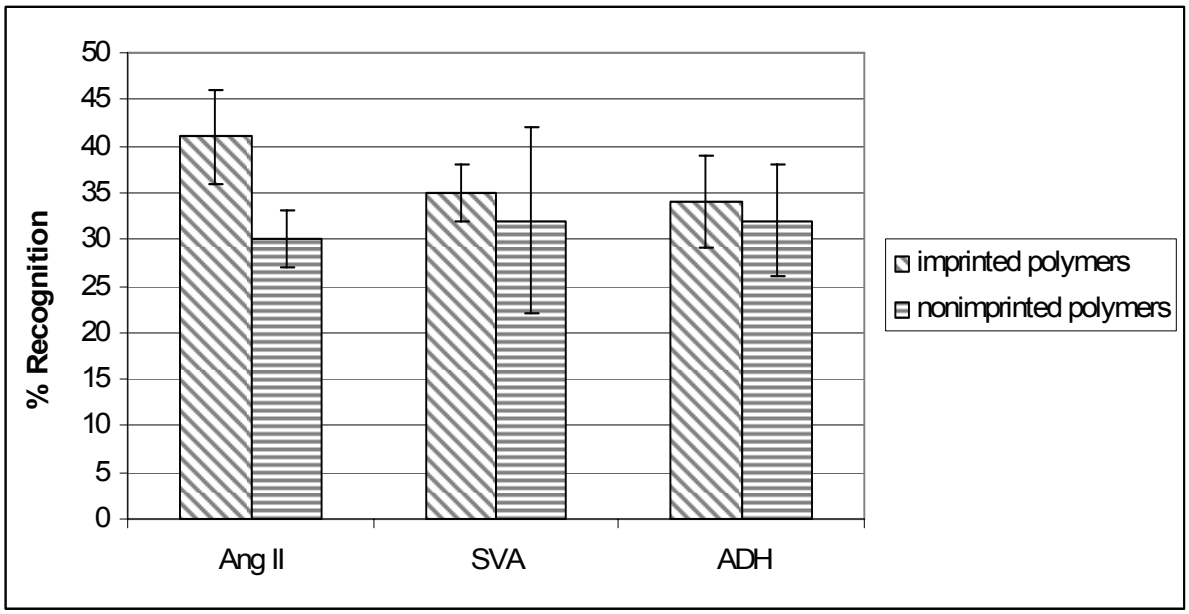

Figure 4.7: Specific Recognition of Imprinted and Nonimprinted Polymers in PBS

Recognition studies performed with pegylated poly(acrylamide-co-methacrylic acid) (PAM) networks with a 1:8 template to functional monomer molar ratio and a crosslinking percentage of 40 mol%. Imprinted polymer was placed in a 1 mg/ml solution of either angiotensin II (AngII), SVA angiotensin (SVA) or ADH and PBS. Figure shows percent of peptide that is bound to the recognitive polymer at 3 h. n=3

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5 CHARACTERIZATION OF NONDEGRADABLE CONFIGURATIONAL BIOMIMETIC IMPRINTED POLYMERS SELECTIVE FOR ANGIOTENSIN II

5.1 Introduction

In creating a synthetic system that mimics naturally occurring recognition processes it is important to understand how the network structure behaves in physiological conditions. The performances of the CIBP networks, in particular, also depend largely on the composition and spatial orientation of the bulk structure. Therefore, it is beneficial to investigate and characterize the structure and functionality of the networks. Examining details such as the macro-porous, micro-porous and non-porous regions of the network, the polymer's free functionalities and its physical response in solution will greatly aid in understanding the performance and will ultimately help in optimizing the system. Investigating the functional group conversion, the crystalline structure and the overall heterogeneity of the network will give insight to the polymers' biocompatibility and long term stability.

Several methods can be used to evaluate the polymer's structure, function and physical response in solution. Namely, the polymers should be analyzed by SEM, FTIR and DSC. Finally, the suitability of hydrogels as biomedical materials and their performance in a particular application depends to a large extent on its bulk

structure. Therefore the porosity of the polymer networks should be evaluated. This can be done by calculating the number-average molecular weight between crosslinks $\overline{M_c}$, network mesh size, ξ, crosslinking density, $\rho_{x,}$ and the equilibrium weight swelling ratio, q, by standard techniques developed in our laboratory.

5.1.1 Scanning Electron Microscopy of Polymers

Scanning electron microscopy (SEM) is a powerful technique that can help discern morphological differences between configurationally imprinted polymers and the nonimprinted controls. The principal means of transport of angiotensin II or template molecules within the recognitive networks is by diffusion through the macromolecular structure. Therefore, an understanding and visualization of the effective spaces in the porous network could help to control this process.

SEMs are able to achieve visualization of porous networks by using a high voltage field emission gun. The electron stream emitted by this gun forms a single beam and then focuses on the sample stage. The instrument then passes the beam through scan coils. When the beam strikes the surface of the sample, it interacts with atoms which in turn emit secondary electrons, primary backscattered electrons, Auger electrons and characteristic X-rays. It is the secondary electrons that are able to give the surface features of the sample.

5.1.2 FTIR Analysis of Imprinted Polymers

Fourier transform infrared spectroscopy (FTIR) can be used to identify chemical compounds which are present in polymer structures. In the case of the CIBPs, it is important to show that the functional monomer functionalities are present in the final polymer network. This information can be used to understand and optimize binding sites and overall recognition.

In imprinted networks, FTIR spectroscopy has been used to determine the nature of the template-monomer complex for small molecular weight drugs and for determining the change in composition of polymers that are templated [**1,2**]. FTIR spectroscopy also has the potential to show the difference between imprinted polymer networks without the presence of template and the networks that contain bound molecules [**3**].

FTIR uses the intrinsic properties of covalent bonds and their thermal energy vibrations to provide useful information about the structure and function of polymers. The molecular vibrations contain characteristic quantized vibrational energy levels that can absorb infrared radiation with energies that correspond to vibrational energy level transitions. The bonds of functional groups, such as carboxyl and amine groups, absorb infrared radiation at characteristic energies and can be easily identified. The most significant portion of the FITR is the frequency range from 1200 cm^{-1} and 600 cm^{-1}. This is known as the fingerprint

region of the IR spectrum because small changes in the structure of the molecule can significantly impact the location and shape of the absorption peaks in this range. By identifying characteristic peaks in this range, the spectrograms can provide powerful insight into the overall chemical compounds present in a particular sample.

5.1.3 DSC Analysis of Imprinted Polymers

Differential Scanning Calorimetry (DSC) is a technique that measures the temperatures and heat flows associated with transitions in various materials as a function of time and temperature in a controlled environment. It can provide quantitative and qualitative information about the physical and chemical changes in the polymeric network. The instrument records endothermic and exothermic changes which provide insight into the melting, recrystallization, and decomposition of polymers. In CIBP networks the DSC can provide important information about reaction kinetics, conversion of potentially toxic functional monomers and glass transition temperatures. These results in turn provide useful information about the overall biocompatibility and long-term stability of the network.

5.2 Materials and Methods

5.2.1 Preparation of Imprinted Polymer for SEM

Polymers were prepared for SEM analysis by cutting swollen, wet polymers into disks with a 12 mm diameter. One corner of the polymer was then sectioned at a 45° angle. This was done in order to image the morphology of the inside of the polymers and not the surface layer which is smoothed during polymerization between two glass plates. The disks were then dried in a vacuum oven at a temperature of 35 °C for two days. The disks were then placed on an SEM stage and sputter coated with a AU/Pd target for 30 sec. The coating was approximately 22 nm thick. The samples were then analyzed by SEM (Hitachi S-4500 field emission scanning electron microscope) at 10 kV.

5.2.2 Preparation of Imprinted Polymer for FTIR

The polymers were prepared for spectroscopic analysis by crushing the dry polymer films to particle sizes ranging from 75-150 μm. A mg of crushed polymer was then placed with 150 mg of dry KBr (Sigma-Aldrich, St. Louis Missouri). This mixture was then compressed into a pellet. A KBr pellet that did not contain polymer was also made as a control. The pellets were then run through a FTIR spectrophotometer (Infinity Gold Series Spectrometer, Mattson, Wisconsin). The final spectra were obtained using 128 scans with a resolution of 1 cm^{-1}. FTIR spectroscopy was run on both the imprinted polymers and the nonimprinted controls.

5.2.3 Preparation of Imprinted Polymer for DSC

The DSC was used to run kinetic studies and determine the glass transition temperatures of the recognitive polymers. All of the kinetic studies were run using the TA 90 differential scanning calorimeter as a miniature reactor (TA instruments, New Castle, Delaware). A diagram of the experimental setup can be seen in Figure 5.1. First, approximately 4.5 to 6.0 mg of the monomer sample was weighed using a digital scale and pipetted into a small aluminum pan using a volumetric pipette. The volume of sample added was approximately 3 µL. The sample pan was then sealed with a piece of polyethylene and placed into the calorimeter. An empty aluminum reference pan was sealed with polyethylene and placed beside it.

Before the monomer was initiated, the atmosphere was made inert by purging the system with nitrogen gas at a pressure of 20 psia for 20 minutes. Then, UV light with an intensity of 1.0 mW/cm^2 was applied to the system for 699 seconds. The heat produced by the reaction with reference to the empty pan was collected over the period of time. Three data points were collected for each second of passing time.

For the glass transition experiments, approximately 5 mg of polymer sample was placed into an aluminum pan. The pan was then sealed and crimped with an aluminum lid. The pan was placed into the calorimeter and a reference pan was placed beside it. The atmosphere was then made inert by purging with nitrogen gas. The DSC scans were started at 30 °C and increased to 120 °C at a rate of 10 °C/min.

All DSC scans were run using four different formulations of polymers that were previously determined to show the best recognition. The four different formulations were divided into two sets of polymers, poly(ethylene glycol)-g-acrylamide (PA) and pegylated poly(acrylamide-co-methacrylic acid) (PAM). Within each set of two polymers, an imprinted polymer which contained Angiotensin II as a template and a control polymer which did not contain the template molecule were made. The polymers all contained a crosslinker percentage of 40 mol% and a template to functional monomer molar ratio of 1:8.

5.3 Results and Discussion

5.3.1 SEM Analysis of Imprinted Polymers

Imprinted and nonimprinted polymer networks were prepared and analyzed by scanning electron microscopy. SEM micrographs of both the imprinted polymers and the non imprinted controls can be seen in Figures 5.2 and 5.3. The polymer

formulations are pegylated poly(acrylamide-co-methacrylic acid) with a crosslinker percentage of 40 mol% and a template to functional monomer molar ratio of 1:8. Figure 5.2 was taken on the Hitachi S-4500 field emission scanning electron microscope. Figure 5.3 was taken on a LEO 1530 scanning electron microscope at a slightly lower magnification of 40.88K x. The results clearly show a morphological difference between the imprinted and non-imprinted polymers. In Figure 5.2 the imprinted polymer is seen having a greater amount of pores and disorganized striations. The pores range from 10-150 nm. In figure 5.3 there is a distinct surface roughness change with the imprinted polymer having a much more varied roughness. The peaks on the surface of the imprinted polymers are located approximately 80 nm apart. These peaks cannot be seen on the non-imprinted controls.

Due to the addition of a template molecule in the imprinted network, one would assume that the imprinted networks would be more porous. This is in part because the functional monomers interact with the template forming cavities around it. In fact, there are several reports in the literature that show morphological differences between imprinted and non-imprinted networks [**4, 5**]. Spizzirri et al. were the first to calculate nanocavitiy size in imprinted networks. It was concluded from these studies that cholesterol imprinting left a size of 15-37 Å. However, it was also noted that higher template loading and the use of porogens could affect the microporosity and lead to higher pore sizes. In the

case of angiotensin II, however, the hydrodynamic radius is estimated to be less that 1 nm [**6**]. This size would not be seen in a typical SEM micrograph, indicating that there are other interactions that are contributing to the more porous network. In particular, acrylamide is known to form polymer loops upon crosslinking when in the presence of a hydrophilic moiety [**7**]. The template molecule angiotensin II could be considered as such a moiety, as it is a hydrophilic peptide.

The visible nanocavities could also be formed due to polymer/template thermodynamic interactions. In short, the solubility of the template angiotensin II in the prepolymerization mixture is not considered perfectly soluble. Therefore, the cavities are often formed by several dissolved template molecules [**5**].

In the end, the imprinted networks did show significant morphological differences which could ultimately have an effect on the overall recognition process. The selective binding therefore becomes a complex process with both interactions with free functionalities from the functional monomers and the effects of size exclusion and diffusion through the networks.

5.3.2 FTIR Spectroscopic Analysis of Imprinted Polymers

FTIR spectroscopy was used in order to analyze the molecular structure of the novel imprinted polymers. This technique allowed not only the molecular

structure of the imprinted network to be probed but also addressed possible template-imprinted polymer interactions. The FTIR spectrogram of a pegylated poly(acrylamide-co-methacrylic acid) with a crosslinker percentage of 40 mol% and a template to functional monomer molar ratio of 1:8 can be seen in Figure 5.4. This spectrogram shows that the free functionalities of the functional monomers are present in the final network instead and have not only interacted with each other.

In order to determine that the imprinted polymer contained the acrylamide and methacrylic acid functionality, several peaks should be present. For the acrylamide, in particular, the spectra should show a strong absorption peak at 1673 cm^{-1} (C=O) stretch and its shoulder peak at 1615 cm-1 (N-H bending) [**8**]. These can be seen in Figure 5.4. The methacrylic acid functionality exhibits a peak at 1421 cm^{-1}. This corresponds to the symmetric stretching of the C=O bond of the bound carboxylic acid group. The peak at 1649 cm^{-1} represents the C=O stretching of the free carboxylic acid groups, meaning those with the hydroxyl group still intact. The intensity of this peak is much lower than that of the bound carboxylic acid peak, indicating that majority of the methacrylic acid monomers have self-reacted during polymerization [**9**]. Ultimately, however, both functionalities are still present in the final network which allows for the hydrogen and ionic bonding of several groups in the angiotensin II template.

5.3.3 Thermal and Kinetic Analysis of Imprinted Polymers

A powerful method of analyzing imprinted structures involves measuring the kinetic data of the polymerization reaction in the presence or absence of a template molecule [**10, 11**]. Thermal analysis was therefore used to collect kinetic data. Both the conversion of the monomer into polymer and the rate of polymerization at each point in the reaction were calculated. This was done for the polymerization reactions both with and without the presence of the template. The rate of polymerization, or R_p, was calculated by the theoretical heat of conversion using equation 5.1. In equation 5.1, it was assumed that the amount of heat that would be released from PEGMA 100 and PEGDMA 1000 would be 13.6 kcal/mol [**12**]. The heat of reaction of all methacrylate derivatives can be approximated using this number, which is also the energy necessary to react a double bond. The heat of reaction of acrylamide was approximated to be 18.9 kcal/mol [**12**]. The theoretical heat of reaction was found according to equation 5.1, where ΔH_T is the theoretical heat of reaction, ΔH is the heat of reaction, x is the sample mass and mw_p is the molecular weight of the polymer.

$$\Delta H_T = \Sigma(\Delta H \cdot x \cdot \frac{1}{mw_p} \cdot \text{mole fraction of component}) \qquad (5.1)$$

Then, the rate of polymerization was calculated using equation 5.2.

$$R_p = \frac{\mathrm{W}}{\Delta \mathrm{H}} \tag{5.2}$$

After the rate of polymerization was calculated at each of the data points, the conversions at each of the data points and overall were calculated. The integrated area under the rate of polymerization curve is conversion. Because the data points were taken at such small time intervals, the area under the curve was approximated using rectangles, as seen in equation 5.3 where the subscript *j* represents each data point.

$$\mathrm{conversion} = \sum_{\mathrm{j=1}}^{\mathrm{n}} \left(\mathrm{time}_{\mathrm{j+1}} - \mathrm{time}_{\mathrm{j}}\right)\left(\mathrm{R}_{\mathrm{p,\,j+1}} + \mathrm{R}_{\mathrm{p,\,j}}\right)\left(\frac{1}{2}\right) \tag{5.3}$$

Both the shapes and the heights of the curves for the rates of polymerizations of the four samples were similar. Figure 5.5 is a graph of each sample's rate of polymerization as function of time. As seen in the graph, the PAM samples yielded slightly higher maximum rates of polymerization. The difference in the maximum rate of polymerizations between the PAM control and the PAM test sample was 3.6% with the control being slightly lower. The PA samples yielded slightly lower maximum rates of polymerization. However, the PA control had a maximum rate of polymerization slightly higher than the test sample by 13.5%.

It was also determined that conversions between the polymer control samples and their respective samples with template were similar. The conversions for the control samples were slightly higher; there was a 7.5% difference in conversions between the PAM control and the PAM test sample and a 6.9% difference in conversions between the PA control and the PA test sample. Figure 5.6 is a graph of the overall conversions for the four polymers.

The kinetics of the polymerization reactions were analyzed in order to determine the overall conversion of the potentially toxic monomers and to examine the effect of the template on the polymerization reaction. The results clearly show that the imprinted networks do not achieve as high of a conversion as the control samples due to the presence of the template. This is a similar conclusion to a trend observed by Ward et al [**10**].

5.3.4 Structural Analysis using Thermal Studies

DSC studies were used to determine the glass transition temperature, T_g, which can be used to analyze the molecular structure. The DSC scans were run to collect kinetic data, and thermograms were collected to determine T_g values. The T_g values of the polymer formulations were determined by using the tangent lines at the onset and end of the transition using the half C_p method, giving a T_g value that corresponds to the middle of this energy transition. Figure 5.6 shows an

example of a thermogram of a poly(ethylene glycol)-g-acrylamide-g-methacrylic acid with a 1:8 template to functional monomer ratio and 40% crosslinker.

The value of the T_g for a polymer is important because it affects the mechanical properties at particular temperatures and determines the temperature range in which a material can be used effectively. Several factors affect the T_g. Bond interaction, molecular weight, functionality, branching and chemical structures are all important in determining T_g. It is possible to calculate a theoretical T_g by using equation 5.4

$$T_{g\infty} = w_x T_{gx} + w_y T_{gy} + \ldots \quad (5.4)$$

Tg values were calculated using equation 5.4 which relates the Tg of an ideal blend ($T_{g\infty}$) to the Tg values of their constituent linear polymers [**13, 14**].

In this equation, $T_{g\infty}$ represents the glass transition temperature of an equivalent linear copolymer which would be observed if all crosslinks in the polymer system were severed. W_x and w_y represent the weight fractions of the respective constituent linear polymers such as poly(acrylamide), poly(methacrylic acid) and PEG. The weight fractions can be easily calculated based on the molar feed concentrations of the comonomers. The Tg values of each of the constituents were based on literature reports.

Table 5.1 summarizes the results for the molecularly imprinted and the control structures studied. The experimental values correlate well with the theoretical values. It can also be seen that the glass transition temperatures of the imprinted networks are not significantly different than the non-imprinted network. This suggests that the template does not affect the overall crystalline structure.

This is a significant finding which indicates that the crosslinked structure remains the same in the presence and absence of the template, suggesting that the template does not affect the crosslinking process. Since the template molecules do occupy some of the nanocavity space, this space is simply the "mesh size" of the imprinted networks. This is important in that it supports the idea of calculating the nanocavity size for measurements of the number average molecular weight between crosslinks, $\overline{M_c}$.

5.3.5 Bulk Structure Analysis of Imprinted Polymers

The suitability of hydrogels as biomedical materials and their performance in a particular application depends to a large extent on their bulk structure. As shown by Spizzirri and Peppas [**5**], the nanocavity structure can be determined from the calculation of the value of $\overline{M_c}$ and the associated mesh size, ξ. Thus, the

number-average molecular weight between crosslinks $\overline{M_c}$, network mesh size ξ, crosslinking density, $\rho_{x,}$ and equilibrium weight swelling ratio, q, were calculated using the procedure described below.

The change of chemical potential due to the elastic retractive forces of the polymer chains can be determined from the theory of rubber elasticity. Upon equaling the Gibbs free energy of the elastic contribution to the Gibbs free energy of the polymer/solvent thermodynamic interactions, as described by the Flory-Huggins Theory, an expression for determining the molecular weight between two adjacent crosslinks, $\overline{M}_{c,}$ of an imprinted hydrogel prepared in the absence of a solvent was written as:

$$\frac{1}{\overline{M}_c} = \frac{2}{\overline{M}_n} - \frac{\left(\bar{\upsilon}/V_1\right)\left[\ln\left(1-\upsilon_{2,s}\right)+\upsilon_{2,s}+\chi_1\upsilon_{2,s}^2\right]}{\left(\upsilon_{2,s}^{1/3}-\frac{\upsilon_{2,s}}{2}\right)} \qquad (5.5)$$

Here, $\overline{M}_n$, is the molecular weight of the polymer chains prepared under identical conditions but in the absence of the crosslinking agent, $\bar{\upsilon}$ is the specific volume of the polymer and V_1 is the molar volume of water. Although the polymer was originally prepared in DMSO, this is an appropriate approximation due to the thermodynamic properties at room temperature [**15**]. The Flory polymer-solvent interaction parameter, χ, was calculated as a weighted average

of the values for PMAA (χ = 0.5987) and PEG (χ = 0.55) in water. Such averaging procedures are not usually acceptable in polymer thermodynamics, but are very appropriate here because of the similarity of the parameter values under the conditions of operation [**5**].

It was then possible to use the modified version of the original Flory-Rehner theory for hydrogels prepared in the presence of water. The presence of water effectively modified the change of chemical potential due to the elastic forces. This term must now account for the volume fraction density of the chains during crosslinking. The resulting Peppas-Merrill equation (equation (5.6)) can be used to determine the molecular weight between crosslinks in a imprinted hydrogel prepared in the presence of water.

$$\frac{1}{\overline{M}_c} = \frac{2}{\overline{M}_n} - \frac{\left(\overline{\upsilon}/V_1\right)\left[\ln\left(1-\upsilon_{2,s}\right)+\upsilon_{2,s}+\chi_1\upsilon_{2,s}^2\right]}{\upsilon_{2,r}\left[\left(\frac{\upsilon_{2,s}}{\upsilon_{2,r}}\right)^{1/3}-\left(\frac{\upsilon_{2,s}}{2\upsilon_{2,r}}\right)\right]} \qquad (5.6)$$

Here, $\upsilon_{2,r}$ is the polymer volume fraction in the relaxed state, which is defined as the state of the polymer immediately after crosslinking but before swelling.

An important structural parameter to analyze hydrogels is the amount of space available between macromolecular chains. This space is often regarded as the molecular mesh. Depending upon the size of these nanosized pores hydrogels can be conveniently classified as (i) macroporous (ii) microporous and (iii)

nonporous. A structural parameter that is often used in describing the size of the pores is the correlation length, ξ, which is defined as the linear distance between two adjacent crosslinks and can be calculated using the following equation:

$$\xi = \alpha \left(\bar{r}_o^2 \right)^{1/2} \tag{5.7}$$

Here, α is the elongation ratio of the polymer chains in any direction and $\left(\bar{r}_o^2 \right)^{1/2}$ is the root-mean-square, unperturbed**,** end-to-end distance of the polymer chains between two neighboring crosslinks. For an isotropically swollen hydrogel, the elongation ratio, α, can be related to the swollen polymer volume fraction, $\upsilon_{2,s}$, using equation (5.8).

$$\alpha = \upsilon_{2,s}^{-1/3} \tag{5.8}$$

The unperturbed end-to-end distance of the polymer chain between two adjacent crosslinks can be calculated using equation (5.9), where C_n is the Flory characteristic ratio ($C_{n,\ PMAA}$= 14.6 and $C_{n,\ PEG}$ = 3.8 [**16**]), l is the length of the bond along the polymer backbone (for acrylic polymers 1.54 A) and N is the number of links per chain that can be calculated by equation (5.10).

$$\left(\bar{r}_o^2 \right)^{1/2} = l \left(C_n N \right)^{1/2} \tag{5.9}$$

$$N = \frac{2\overline{M}_c}{M_r} \tag{5.10}$$

In equation (5.10), M_r is the molecular weight of the repeating units from which the polymer chain is composed. Finally, when one combines equations (5.7) through (5.10), the correlation distance between two adjacent crosslinks in a swollen hydrogel can be obtained:

$$\xi = \upsilon_{2,s}^{-1/3}\left(\frac{2C_n\overline{M}_c}{M_r}\right)^{1/2} l \tag{5.11}$$

The bulk structure characteristics are summarized in Table 5.2. The mesh size of the polymer is approximately 13 Å. The hydrodynamic radius of angiotensin II is estimated to be less that 1 nm [**6**] which is less than 10 Å. Therefore, the template molecule should be able to move throughout the network without much hindrance.

5.4 Conclusions

Spectroscopic analysis and structural investigation provided important information about the imprinted networks and the effects that the polymerization process has on them. Most significantly, the presence of a template during the polymerization process has negligible effects on the conversion of the functional

monomers and the crosslinking process. This is important because unconverted monomer can have future effects on the cytotoxicity of the gel. The polymerization process also did not affect the presence of free functionalities of the functional monomers in the final network. FTIR spectroscopy was able to confirm that all functionalities were present in the final gel form.

Although the polymerization process was basically unaffected by the presence of a template molecule, the overall morphology of the imprinted network was. SEM micrographs showed significant morphological differences between the imprinted polymers and the non-imprinted controls. This is consistent with previous work by Spizzirri et al. [**5**] which suggests that this is due to polymer/template thermodynamic interactions. In this case the template is not completely soluble and can form aggregates which increase the pore size in the networks.

Ultimately the results of this work provide useful information about the general biocompatibility and long-term stability of the network. They also provide valuable insight to the function of the network which can be beneficial for optimizing the overall recognition process.

5.5 Figures

A – Nitrogen gas inlet
B – UV light source
C – Reference pan
D – Pan with sample
E – Differential photocalorimeter

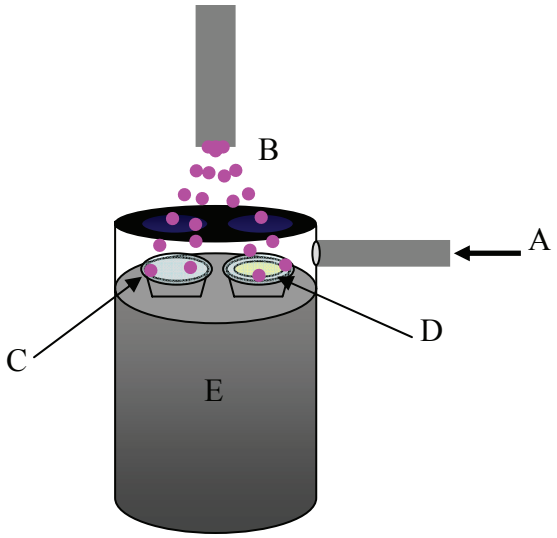

Figure 5.1: DSC Experimental Apparatus

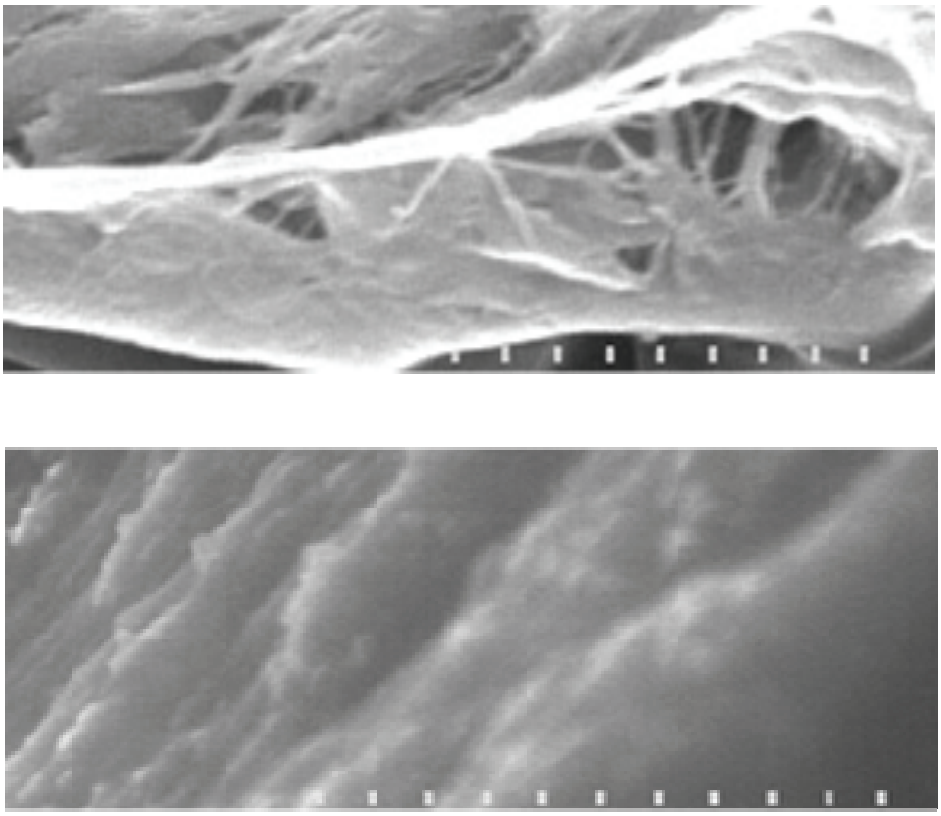

Figure 5.2: SEM analysis
SEM analysis of pegylated poly(acrylamide-co-methacrylic acid) (PAM) networks with a 1:8 template to functional monomer molar ratio and a crosslinking percentage of 40 mol% (top) and control non-imprinted polymer (bottom). Pore sizes of imprinted networks are 10-150 nm. Scale bar = 3.00μm

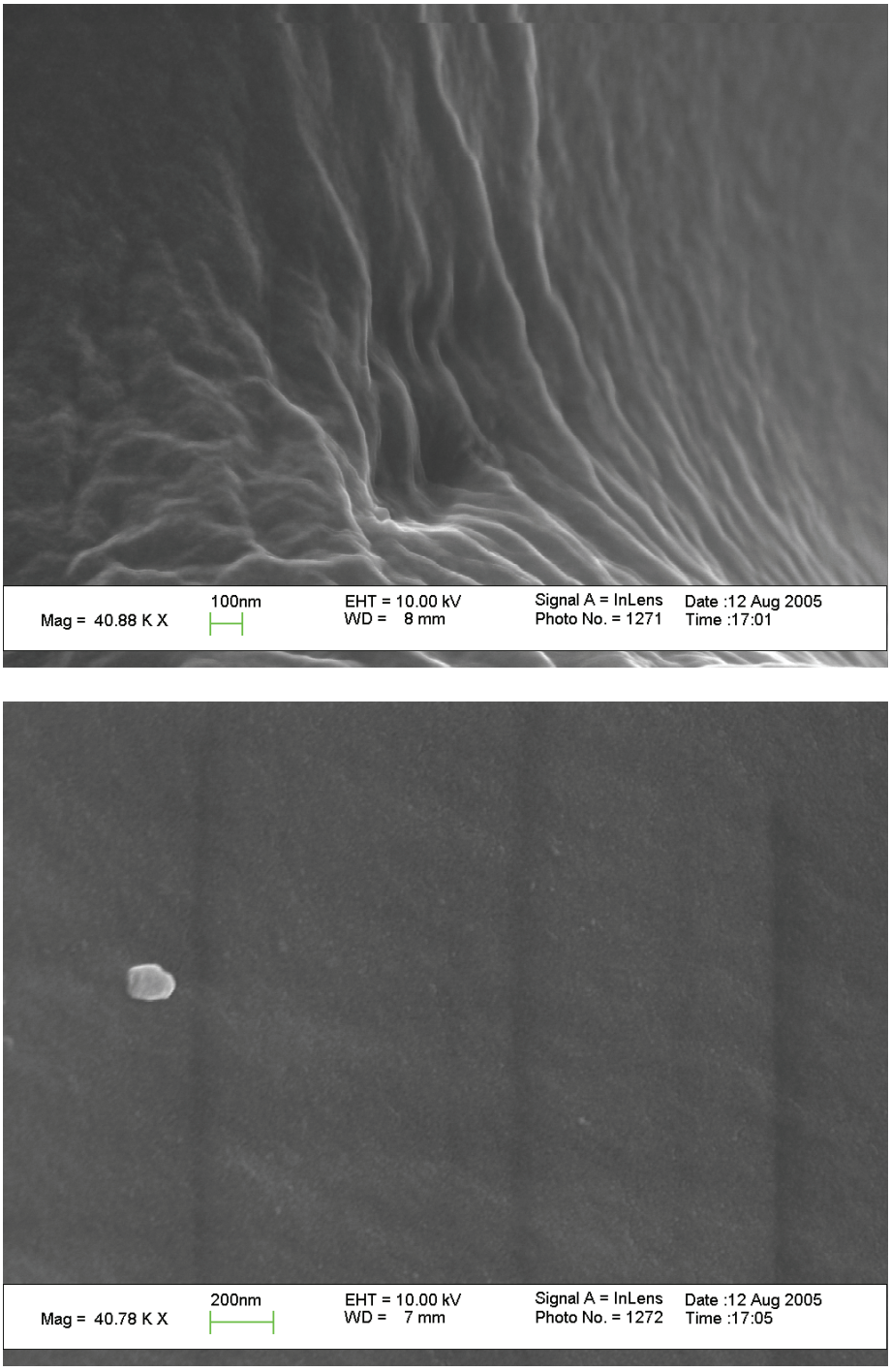

Figure 5.3: SEM analysis
SEM analysis of pegylated poly(acrylamide-co-methacrylic acid) (PAM) networks with a 1:8 template to functional monomer molar ratio and a crosslinking percentage of 40 mol (top) and control non-imprinted polymer (bottom).

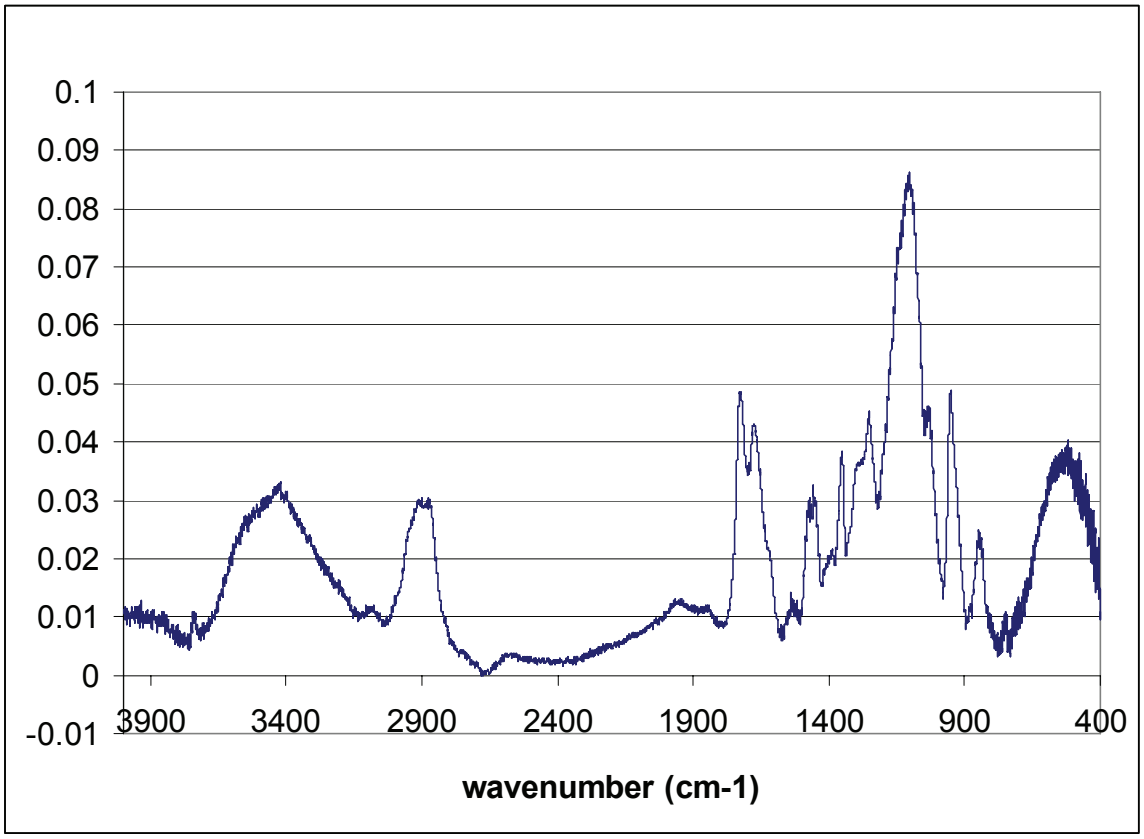

Figure 5.4: FTIR spectrum of CIBP
FTIR spectra of pegylated poly(acrylamide-co-methacrylic acid) (PAM) networks with a 1:8 template to functional monomer molar ratio and a crosslinking percentage of 40 mol%.

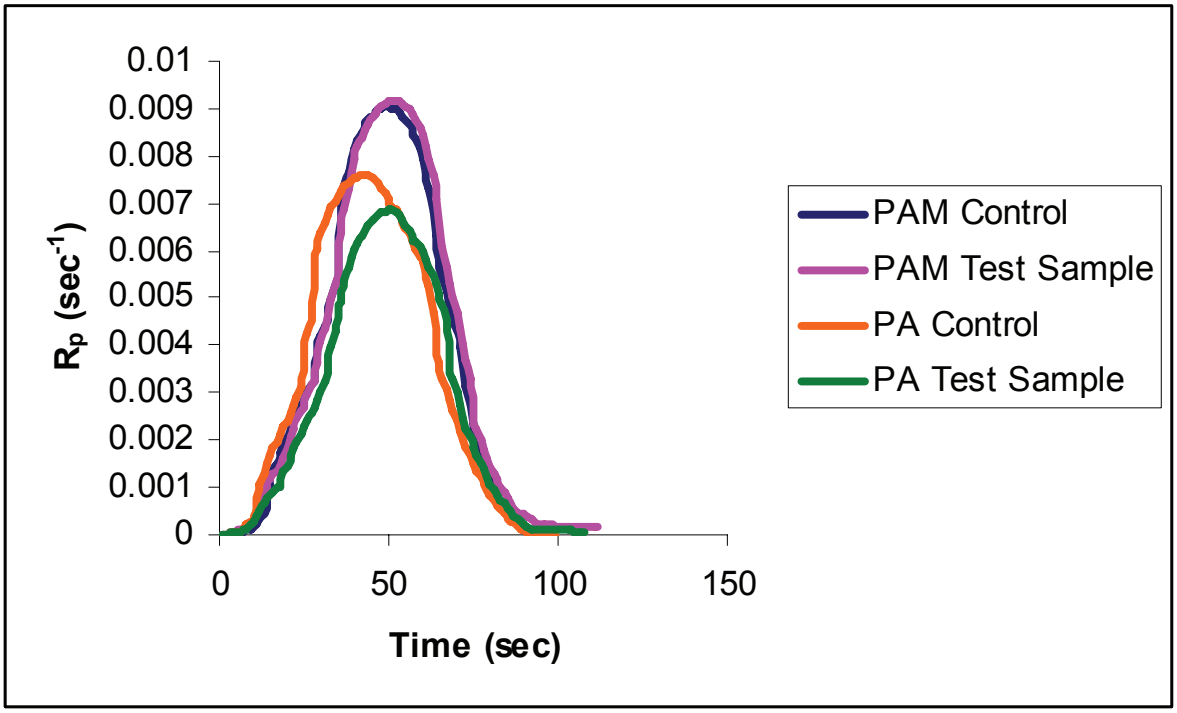

Figure 5.5: Rates of Polymerization versus Time
Samples of pegylated poly(acrylamide-co-methacrylic acid) (PAM) networks with a 1:8 template to functional monomer molar ratio and a crosslinking percentage of 40 mol%, poly(ethylene glycol-g-acrylamide) (PA) networks with a a 1:8 template to functional monomer molar ratio and a crosslinking percentage of 40 mol% and their nonimprinted controls.

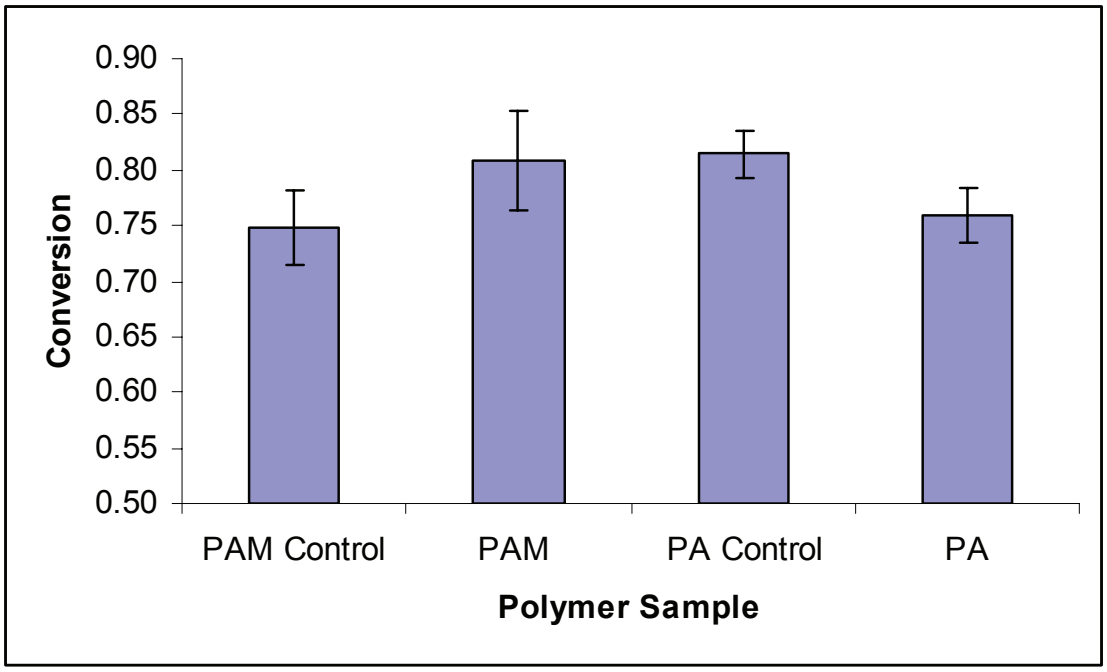

Figure 5.6: Overall Conversions
Samples included pegylated poly(acrylamide-co-methacrylic acid) (PAM) networks with a 1:8 template to functional monomer molar ratio and a crosslinking percentage of 40 mol%, poly(ethylene glycol-g-acrylamide) (PA) networks with a 1:8 template to functional monomer molar ratio and a crosslinking percentage of 40 mol% and their nonimprinted controls.

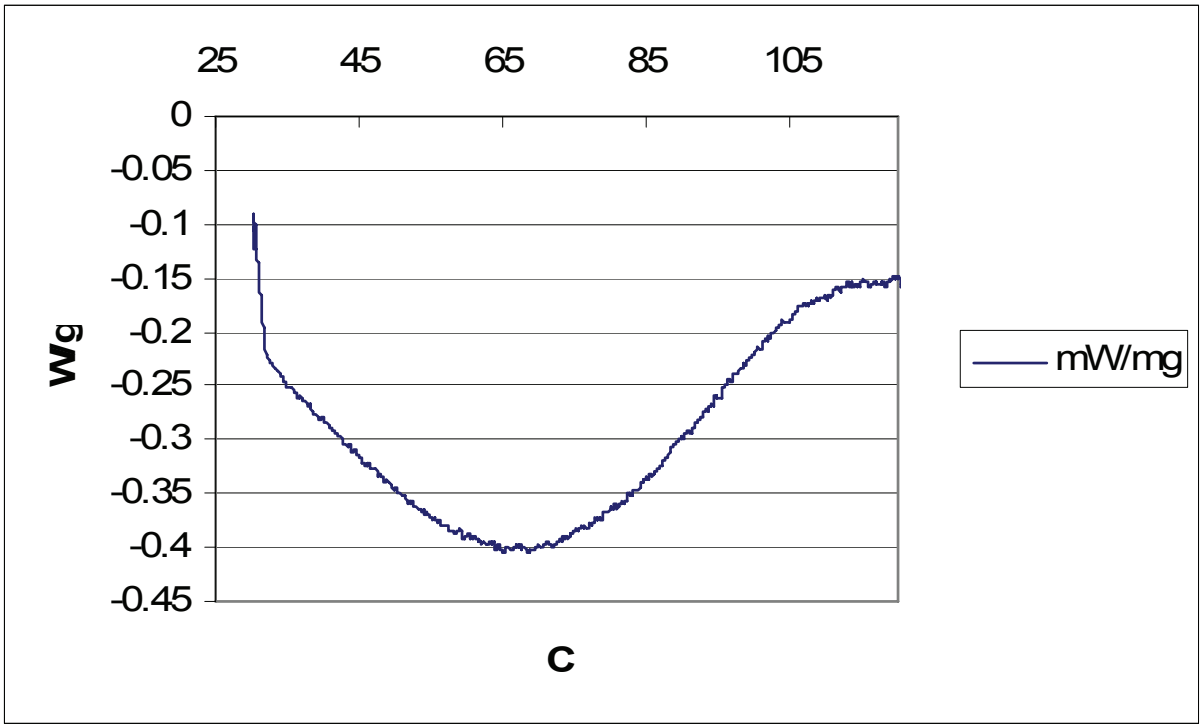

Figure 5.7: Thermogram of Poly(ethylene glycol)-g-acrylamide-g-methacrylic acid imprinted polymer

Tg is 71.62 °C

Table 5.1: Observed and Theoretical Tg of Polymer Formulations

Polymer Formulation	$T_{g,\ observed}$ °C	$T_{g,\ theoretical}$ °C
P(EGMA 400)	n/a	-59
P(Aam)	n/a	165
P(MAA)	n/a	228
P(EGDMA 1000)	n/a	141
PA control	68	58.44
PA imprinted	67.88	55.7
PAM control	73.13	81.95
PAM imprinted	71.62	77

Table 5.2: Bulk Structure Characteristics

Polymer	Equilibrium Polymer Vol Fraction, $\upsilon_{2,s}$	Molecular Weight Between Crosslinks, $\overline{M_c}$ ($g \cdot mol^{-1}$)	Mesh size, ξ (Å)	Equilibrium weight swelling ratio, q
PA Control	0.443	103.548	12.57	3.8
PA Imprinted	0.441	94.687	12.67	2.88
PAM Control	0.229	112.41	12.89	3.5
PAM Imprinted	0.215	125.581	13.59	2.56

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6 FABRICATION AND CHARACTERIZATION OF DEGRADABLE CONFIGURATIONAL BIOMIMETIC IMPRINTED POLYMERS SELECTIVE FOR ANGIOTENSIN II

6.1 Introduction

Although significant advances have been made in molecularly imprinted networks during the past two decades, there has not been much development of them for use in the drug delivery field. This is in part due to the fact that in order to successfully use MIPs for many applications in pharmaceutical treatments, DDS and tissue engineering constructs, the networks themselves need to have a biodegradable component. Having the constructs or devices degrade allows for sustained release of drug and complete removal of the foreign object from the physiological environment without the need for further invasive procedures.

Several biodegradable materials have been studied extensively for their use in biomedical applications such as temporary tissue scaffolds, barriers for surgical adhesions and matrices for DDS [**1-7**]. These materials can usually be classified into three categories of degradation: solubilization, enzyme catalysis and/or chemical hydrolysis [**8**]. In all of these cases it is important that the polymer constructs have the appropriate water permeability, biocompatibility and tensile strength. It would also be ideal if the polymer left no residual polymer after degradation and no toxic by-products [**7**]. For many devices it is important that

the degradation rate be tuned and adjusted to the requirements of the application.

Perhaps, the most common synthetic biodegradable polymers that are being investigated are poly(α-hydroxy esters), including poly(glycolic acid) (PGA), poly(lactic acid) (PLA), their copolymer poly(lactic-co-glycolic acid) (PLGA) and poly(ε–capralactone) [**9**]. Primarily, this is because they are FDA approved and already clinically used. They are considered biocompatible and their degradation products are not cytotoxic. PLA and PGA also can be easily processed and their degradation rates and physical and mechanical properties can be adjusted over a wide range simply by using various molecular weights and copolymers. The chemical properties of poly(α-hydroxy esters) allow hydrolytic degradation through de-esterification. Once degraded, the monomeric components of each polymer can be removed by natural pathways. In fact, the body already has highly regulated mechanisms for completely removing the monomeric components of lactic and glycolic acid [**10**].

In configurationally imprinted biomimetic polymers that recognize peptides and proteins, the poly(α-hydroxy ester) degradable components can also potentially degrade or denature proteins or peptides which are entrapped in the network. This depends, however, on the degree of crosslinking and the amount of acid available. This process can take place because many amino acid chains are

acid labile. Therefore, in a microenvironment, such as a recognitive network where the peptide or protein is bound, the acidic by-products of poly(α-hydroxy esters) can have catastrophic effects on the peptide by cleaving it and rendering it inactive and unable to interact with its receptors. This can be an advantageous side effect when it is desirable to remove excess proteins or peptides from the body. Situations like this occur when the body upregulates the particular protein and it becomes over-expressed. Systems that are capable of achieving this effect could become part of a whole new generation of therapeutic systems.

It was, therefore, a goal of this work to incorporate a poly(α-hydroxy ester) degradable crosslinker into the recognitive network. By only replacing the crosslinker, and replacing the crosslinker with a structurally similar one, it was hoped that the new recognitive network would show very little compromise to the original recognition abilities of the nondegradable network. Ultimately, this new system would also allow for a more biocompatible system that is easily cleared from the body and possibly provide for a novel drug delivery device by being able to destroy bound oligopeptides.

6.2 Materials and Methods

6.2.1 Preparation of Hydrolytically Cleavable Poly(α-hydroxy ester) Crosslinkers.

Poly(α-hydroxy ester) crosslinkers were synthesized according to a method originally described by Sawhney et al. [**10**] In a characteristic experiment, 30 g of dry α,ω-dihydroxy PEG 1000 (Sigma-Aldrich, St. Louis, Missouri), 3.6 g of glycolide (E. I. du Pont de Nemours) and 15 mg stannous octoate (Sigma-Aldrich, St. Louis, Missouri) were charged into a round-bottomed flask under a nitrogen atmosphere. The mixture was then stirred under vacuum at room temperature for 1 hour. It was then heated to 160°C under nitrogen for 16 hours and finally allowed to cool to room temperature. In order to switch between nitrogen and vacuum a dual bank manifold was used (Figure 6.1). The copolymer that was created was dissolved in dichloromethane, precipitated in anhydrous ether and dried.

The pegylated poly(glycolic acid) was then end-capped with acrylate groups in order to form a photopolymerizable polymer. This was synthesized by dissolving 30 g of pegylated poly(glycolic acid) in 300ml of dichloromethane. The mixture was then cooled to 0°C. While maintaining that temperature, 1.30 ml of triethylamine and 1.58 ml of acryloyl chloride (Sigma-Aldrich, St. Louis, Missouri) was added. This was then stirred for 12 hours at 0°C and 12 hours at room temperature. The mixture was filtered to remove triethanolamine hydrochloride. The macromer was ultimately captured by pouring the filtrate in dry diethyl ether. This was finally purified by dissolution and reprecipitation using dichloromethane and hexane. It was then dried under vacuum at 70°C.

The final product of this synthesis produces a pegylated poly(glycolic acid) diacrylate macromer which can act as a crosslinker and be photopolymerized into a polymer. This can then be hydrolytically cleaved and degrade into PEG, glycolic acid and poly(acrylic acid). The overall reaction scheme of this synthesis can be seen in Figure 6.2.

6.2.2 Preparation of Pegylated Poly(glycolic acid) Diacrylate Macromers for FTIR Analysis

FTIR spectroscopy was conducted in order to characterize the macromers. The FTIR pellets were prepared by crushing the dry macromers at all stages of synthesis and purification to particle sizes ranging from 75-150 μm. A sample of 1 mg of crushed macromer was then placed with 150 mg of dry KBr (Sigma-Aldrich, St. Louis Missouri). This mixture was then compressed into a pellet. The final PGA-PEG-PGA diacrylate macromer was waxy and could not be crushed. It was, therefore, dissolved in dichloromethane and added dropwise with a Pasteur pipette into 150 mg of dry KBr. The KBr/macromer mixture was then mixed and allowed to dry in a 70°C vacuum oven overnight. A KBr pellet that did not contain macromer was also made as a control. Pellets were also made for the original α,ω-dihydroxy PEG 1000 for comparison. The pellets were then run through a

FTIR spectrophotometer (Infinity Gold Series Spectrometer, Mattson, Wisconsin). The final spectra were obtained using 128 scans with a resolution of 1 cm^{-1}.

6.2.3 Synthesis of Degradable Imprinted Polymer

The newly synthesized PGA-PEG-PGA 1000 diacrylate was used as a degradable crosslinker and substituted for the poly(ethylene glycol) 1000 dimethacrylate crosslinker in the nondegradable network. The recognitive polymers were then prepared in an identical way to the nondegradable systems. The most recognitive nondegradable network formulation was used for the degradable systems. This was poly(ethylene glycol) grafted poly(acrylamide-co-methacrylic acid) with a crosslinker percentage of 40 mol% and a template to functional monomer ratio of 1:8.

Briefly 0.095g of (PEGMA 400) (Polysciences Warrington, PA.), 0.15 g of methacrylic acid (MAA), and 0.13 g of acrylamide (AAm) (Sigma-Aldrich, St. Louis Missouri) were placed in an amber bottle and dissolved in 0.6707 g DMSO (Fisher Scientific). The monomers were in a 1:1:1 molar feed ratio of ethylene glycol units:AAm:MAA. Then, 0.043 g of angiotensin II was added. The solution was allowed to sit for 20 minutes in order to form prepolymerization complexes. A sample of 0.2255 g of pegylated poly(glycolic acid) 1000 diacrylate crosslinker

was then added. After the monomer solution was mixed and allowed to go into solution, the photoinitiator 1-hydroxyclyclohexyl phenyl ketone (Irgacure 184®) (CIBA-GEIGY Hawthorne, NY) was added.

The amber bottle was then covered with parafilm and placed in a nitrogen atmosphere. A needle was placed through the parafilm and nitrogen was allowed to bubble through the solution for 20 minutes in order to remove the oxygen, a free-radical scavenger. The solution was then pipetted between two glass plates which were separated by a 700 μm Teflon spacer. The glass plates were then exposed to UV light (Dymax 2000_EC Light Curing System, Torrington, CT) for 20 minutes at an intensity of 16 mW/cm^2. The resulting polymer was then removed from the glass plates and washed in a 300ml solution of acetonitrile with 5% acetic acid for 7 days. This was to ensure template extraction and the removal of any unreacted monomer. The polymer was then dried in a vacuum oven at 35°C for 2 days. It could then be cut into 12 mm diameter disks or crushed into particles ranging in size from 75-150 μm. Control polymers were synthesized in the same manner with the exception that no angiotensin II was added.

6.2.4 Recognition Studies of Degradable Imprinted Polymer

To determine if angiotensin II was successfully imprinted into the polymer networks, recognition studies were performed. The polymer was placed in the presence of angiotensin II and SVA angiotensin. SVA angiotensin is a synthesized derivative of angiotensin II in which three amino acid moieties were substituted. Its structure is **ser**-arg-val-tyr-**val**-his-pro-**ala.** It has a molecular weight of 958 and a charge at pH 7 of +1. Both of these characteristics are similar to angiotensin II. The binding studies were also conducted in both DMSO, the solvent used during the synthesis, and phosphate buffered solution (PBS) with a pH of 7.4. The latter was used to simulate physiological conditions.

In a typical experiment, dry imprinted polymers were cut into 12 mm diameter disks and weighed. A disk was then placed into a 15 ml conical tube which had either solutions of 1 mg/ml angiotensin II in DMSO, 1 mg/ml angiotensin II in PBS, 1 mg/ml SVA angiotensin in DMSO or 1 mg/ml SVA angiotensin in PBS. Nonimprinted control polymers of the same formulation were also cut into 12 mm disks, and placed into their own 15 ml conical tubes containing the solutions described above. The tubes were then placed on a rotating mixer set at 25 rpm. At time points of 0, 1, 2, and 3 hours, three 200 µl samples of supernatant were removed and placed into a UV/Vis 96-well plate. The plates were then read by a UV/Vis microplate reader at a wavelength of 215 nm. The results were then compared to standard curves of known concentration of both angiotensin II and SVA angiotensin. From this the peptide concentration in the supernatant was

determined. This concentration was then subtracted from the original solution concentration. The final result was then divided by the weight of the original dry polymer to determine mg bound/mg polymer. All experiments were repeated in triplicate for each formulation of the polymer.

The degradable polymer construct was then tested for specific recognition against a physiologically relevant peptide. The binding studies were performed as previously described; however, instead of using SVA angiotensin, a synthesized peptide which is also present in the blood stream, antidiuretic hormone (ADH) was used.

6.2.5 In Vitro Degradation

To measure the degradation rates of the degradable construct, imprinted and non-imprinted polymers were cut into 12 mm diameter disks and dried in a vacuum oven for 24 hours. The disks were then equilibrated in 100 ml of PBS at pH 7.4. The disks were then weighed to determine the initial polymer weight. The polymers were returned to the PBS, placed on a shaker plate and incubated at 37°C. Weight loss was measured gravimetrically each day for a period of 3 weeks.

6.2.6 Gel Permeation Chromatography of Degraded Polymers

Degradation products of the degradable polymers were measured by gel permeation chromatography (GPC) in order to determine their molecular weight. In physiological conditions, the molecular weight of the degradation products can influence the body's immune response and determine whether it can be cleared by the RES. Therefore, imprinted and non-imprinted polymers with PGA-PEG-PGA diacrylate macromers incorporated as their backbone were allowed to degrade in PBS at 37°C. The solution was then placed in a rotary evaporator in order to remove excess liquid and concentrate the polymer degradation products. A sample of 50 μl of the concentrated solution was then injected into an Alliance GPC (Waters, Milford MA). A sample containing only the solvent PBS was also run for elution peak comparison. Elution time was then recorded and molecular weight determined.

6.2.7 Placement of Angiotensin II in Degradable Conditions

It is the hypothesis that when a biodegradable component is added to the imprinted system, the acid that accumulates during degradation will have a catastrophic effect on the recognized peptide. Therefore, the following set of experiments was run to determine the extent to which angiotensin II could be cleaved by acid hydrolysis. 100 μg of angiotensin II were placed into 100 μl of

various acids. The acids included 70% lactic acid, 85% lactic acid, 6N HCl, 70% formic acid, 70% THF and 70% glycolic acid. These solutions were then incubated at either 37°C or 70°C for 24 hours. After the incubation period, the samples were run on an electrospray ionization (ESI) mass spectrometer. For comparison, a sample of 1 µg angiotensin II/1 µl H_2O was also run through ESI.

6.3 Results and Discussion

6.3.1 FTIR Analysis of Synthesized Crosslinker

FTIR spectroscopy was conducted at various stages of the synthesis of the new crosslinker in order to ensure the soundness and accuracy of the structure. A spectrogram of the original α,ω-dihydroxy PEG 1000 precursor can be seen in figure 6.3. The key peaks of the pure PEG that can be seen are the 2880 cm-1 due to the C-H stretch, an 1110 cm-1 due to the ether stretch and the absorbance at 3510 cm-1 due to the terminal hydroxyl group. After the addition of the poly(glycolic acid) groups, a FTIR spectrogram should show an additional absorbance peak at 1756 cm-1. This shows the presence of an ester stretch due to glycolidyl moieties. This is seen in Figure 6.4. Finally, it is the free terminal hydroxyl group that reacts with the acrylate groups during acrylation. Therefore, after the addition of acrylate groups to the pegylated poly(glycolic acid), there should be a loss of absorption at 3510 cm-1 [**11**]. This can be seen in Figure 6.5. Ultimately, a purified poly(α-hydroxy ester) crosslinker spectrogram should show

strong absorbance peaks in the ester stretch, the ether stretch and the C-H stretch. The presence of all of these peaks in Figure 6.5 ensures that the crosslinker was successfully synthesized and that the chemical structure is sound.

6.3.2 Recognition Analysis of Degradable Imprinted Polymer

After the incorporation of the synthesized crosslinker into the recognitive network, the newly formed degradable CIBPs were compared to the nondegradable networks. The polymers were visually compared and recognition studies were performed to ensure that the recognitive abilities of the system were still intact. Upon visual inspection the polymer networks appeared relatively similar with both gels being fairly transparent.

Of greater importance than the comparison of gels' visual appearances was the comparison of their recognitive abilities. Recognition studies were, therefore, performed on the degradable network. The imprinted polymer was compared both to its non-imprinted control and the competitive peptides SVA angiotensin and the biologically relevant ADH for specific recognition. The results of these studies are shown in Figure 6.6.

The degradable network, compared to the nondegradable network, showed very little loss in recognition capabilities. Although is showed slightly less specific recognition of angiotensin II versus its competitive peptides, it was approximately 23% more selective for angiotensin II than SVA angiotensin, and approximately 28% more selective for angiotensin II than ADH. Part of the reason for loss of specificity could be due to the fact that the crosslinker is slightly bulkier than its nondegradable counterpart. It could, therefore, be possible to increase this recognition by shortening the length of the original PEG precursor. However, although this specificity is less than the nondegradable polymer, it still demonstrates that it is possible to add a degradable component to the system and maintain recognition. A selectivity of over 20% for an oligopeptide is also considered relatively high when compared to the findings reported in the current literature [**12**].

The main difference between the degradable and nondegradable network was that when it was compared to its nonimprinted control the degradable polymer was approximately 17% more selective for angiotensin II. This was significantly less than the 26% seen in the nondegradable network. The result implies that the network is binding to molecules based on nonspecific interactions. A possible explanation for this is that the gel contains an excess of free functionalities. Again, if the degradable crosslinker was not fully acrylated with double bonds on each side of its chains, a terminal free hydroxyl group would remain at the end of

the poly(glycolic acid) chain. This hydroxyl group would be free to interact with molecules because the chain would be grafted into the polymer instead of crosslinked. Ultimately this would lead to more hydrogen bonding sites and potentially an increased amount of nonspecific absorption.

6.3.3 Degradation of Polymer Gels

The degradation characteristics of the imprinted gels are particularly important if the polymers are to be used in therapeutic treatments. The degradation of the network is a function of the crosslink density as well as the hydrolytic susceptibility of the polyester. Ultimately, it would be expected to have similar characteristics to the degradation of PLA and PGA.

Several measures can be taken to design and tune the degradation rates. Highly crosslinked networks have slower degradation times than more loosely crosslinked polymers. Also, certain ester linkages are more hydrolytically susceptible than others, with the glycolidyl linkage being the most susceptible. Finally, the inclusion of PEG chains in the network will greatly affect its degradation rate. It has been shown that including PEG will reduce the amount of time it takes the network to degrade. This has been attributed to the fact that PEG is relatively hydrophilic and can solubilize larger molecular weight fragments

that would be soluble for a homopolymer such as PGA. Also, PEG increases the accessibility of water to the polymer matrix which ensures bulk erosion [**11-17**].

The degradation profiles of the imprinted and non-imprinted control polymers, which were subjected to in vitro degradation, can be seen in Figure 6.7. The total time taken for the gels to degrade into completely water soluble products at pH 7.4 and 37°C was 19 days. It should be noted, however, that the gels were 12 mm circular disks with a thickness of 700 μm. It would, therefore, be assumed that if the same polymer formulation was used but was crushed into microparticles, the total degradation time would be much more rapid. It is also interesting to notice that the addition of an imprinted template to the polymer formulation did not exert a significant effect on the degradation rate of the network. The imprinted network had only a slightly more rapid initial rate which is possibly due to it being a more porous structure.

The degradation products of the polymer were also analyzed by GPC. An example of a chromatograph can be seen in Figure 6.8. The solution eluted over a period of 60 minutes. The elution peak at 41.6 minutes is the largest and the only peak that varies from the elution peaks of the blank solvent run and, therefore, corresponds to the polymer degradation products. When compared to the standard PEO calibration curve, this yields a polymer molecular weight of 986. It was not possible to calculate a PDI of the degradation products due to the

fact that that the polymer peaks are imbedded in the solvent peaks. The mw order of magnitude is, however, what one would expect if the polymer completely degraded because the original starting material was the nondegradable PEG 1000.

6.3.4 Analysis of Angiotensin II in Degradable Conditions

The oligopeptide angiotensin II was tested for stability in various acidic conditions in order to determine whether or not acid byproducts from degradable polymers could be used as a therapeutic treatment for removing over-expressed peptides.

The first goal of this work was to show proof of concept that the amino acid links of the peptide could be cleaved in acidic conditions. Therefore, the conditions were varied from extreme to more mild ones. The acids that the peptide was placed in included 70% lactic acid, 85% lactic acid, 6N HCl, 70% formic acid, 70% THF and 70% glycolic acid. The temperature was also varied from 70°C to 37°C. The higher temperature value was selected in order to provide an extreme environment for degradation by acting as a catalyst. The lower temperature was chosen to mimic physiological conditions. It was assumed that if there was no degradation of the peptide at 70°C after analyzing the solutions by mass spectrometry: the peptide would not degrade at 37°C. The time point of 24 hours was chosen as a representative time for a treatment such as this to be

considered viable. Ultimately, the only acidic environment capable of cleaving angiotensin II turned out to be one consisting of glycolic acid. At 70°C mass spectrometry showed complete destruction of the peptide with the original peak of 1046 no longer existing. At 37°C, mass spectrometry showed degradation peaks, but the 1046 peak still remained suggesting that some of the original peptide stayed intact. (Figure 6.9) Overall, however, this shows that it is possible to degrade angiotensin II in a physiological environment with a 70% concentration of glycolic acid.

Beyond showing that angiotensin II can be cleaved, these results are valuable because they give insight to important characteristics of the degradable network. They imply that the degradable crosslinker must contain glycolidyl moieties if it is to be used as a therapeutic treatment for removing excess angiotensin II. This is also promising because PGA degrades relatively faster that its hydrophobic poly(α-hydroxy-ester) cousin PLA, making it more suitable for rapid therapy. This is particularly desirable when trying to control over-expressed peptides that fluctuate on a daily basis as circadian and other period rhythms.

6.4 Conclusions

A poly(α–hyrdoxy ester) diacrylate crosslinker was successfully synthesized and incorporated into a network that is recognitive and selective for angiotensin II.

The FTIR analysis of the crosslinker showed that the correct structure was present but could not give a quantitative analysis of whether there were any impurities, such as nonacrylated chains, remaining. Although the synthesized crosslinker is similar is size, structure and function of the PEG 1000 DMA crosslinker that it is substituting for, there are some differences between the physical and recognitive abilities of the degradable and nondegradable networks. It is possible, however, that left over impurities from the synthesis lead to these differences.

The degradable imprinted polymer is structurally sound and does show approximately a 28% selectivity of angiotensin II over another physiologically available peptide, ADH. It also degrades at a relatively rapid rate due in part to the fact that it has glycolidyl ester linkages. This is significant because if the polymer is to be used as part of a treatment for removing over-expressed peptides, it has to be fast to recognize and quick to degrade. It is also the glycolic acid moieties that would be responsible for acting on or degrading the bound peptides. This is because it has been shown that glycolic acid has the ability to cleave angiotensin II and would render it inactive so as to prevent it from interacting with its receptors.

Ultimately the results of this work are very promising because it is one of the first times molecularly imprinted systems have been shown to recognize a template

and degrade. By developing systems such as these, it is possible to broaden the scope of the molecular imprinting field, ultimately making it possible for imprinted systems to be successfully used in pharmaceutical treatments, drug delivery devices and tissue engineering constructs.

6.5 Figures

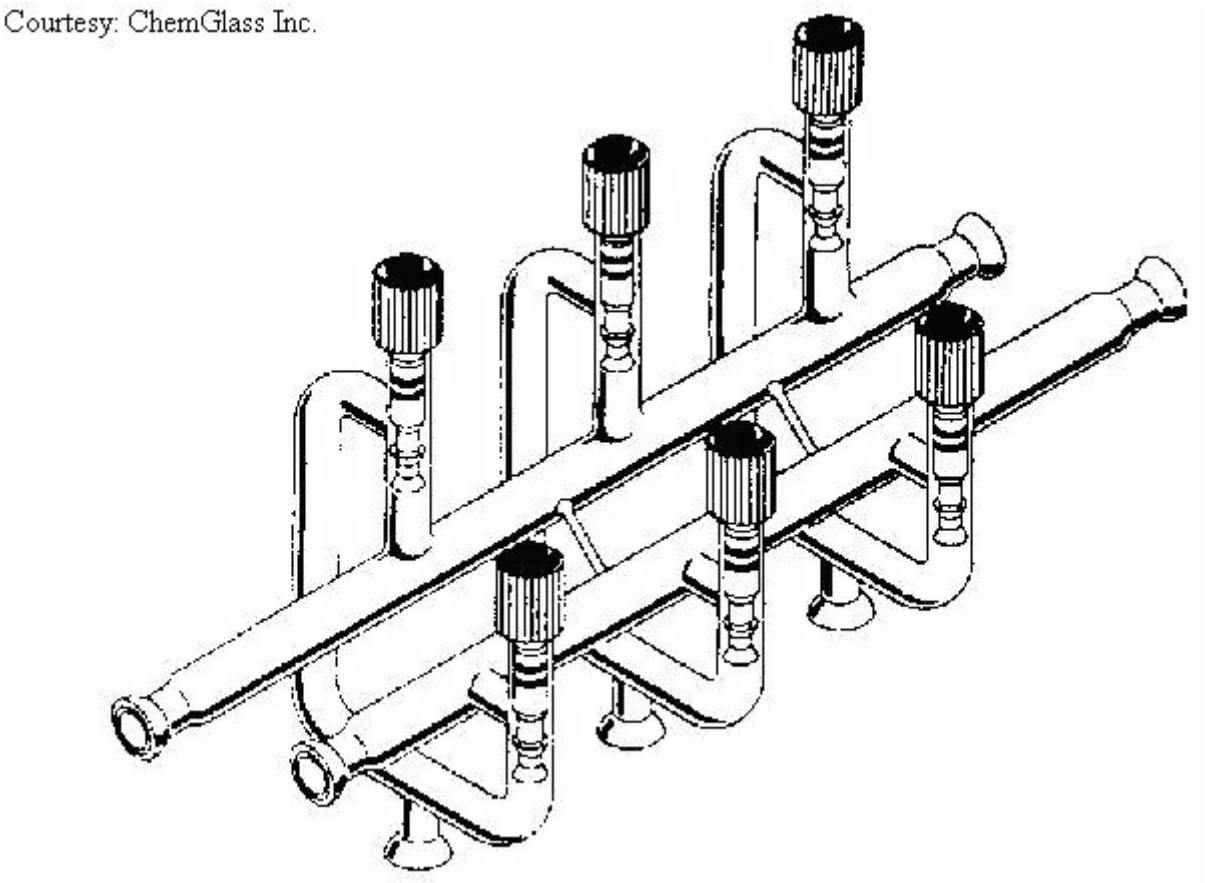

Figure 6.1: Dual Bank Manifold

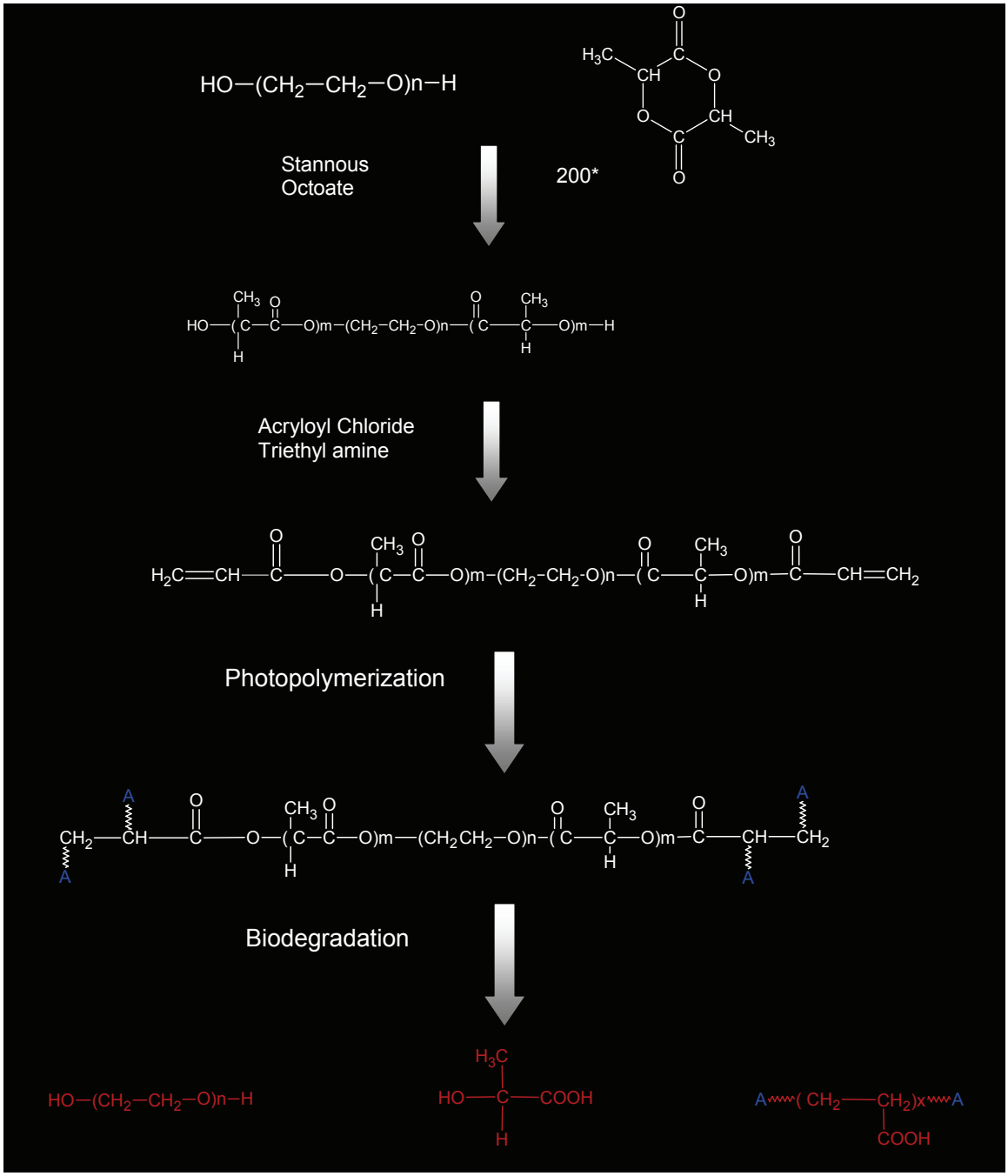

Figure 6.2: Reaction Scheme of Poly(α-hydroxy ester) diacrylate Synthesis and Degradation

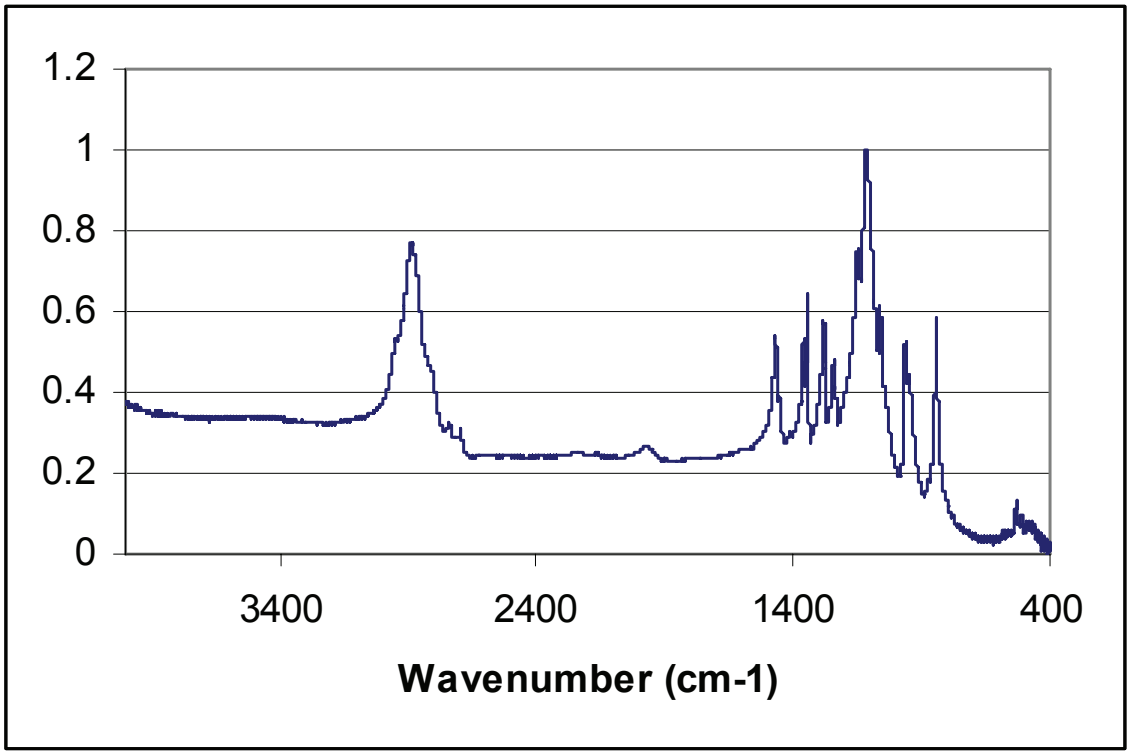

Figure 6.3: FTIR Spectrogram
FTIR spectrogram of α,ω-dihydroxy PEG 1000 precursor. The peak at 2880 cm-1 is due to the C-H stretch, the peak at 1110 cm-1 is due to the ether stretch and the absorbance at 3510 cm-1 is due to the terminal hydroxyl group.

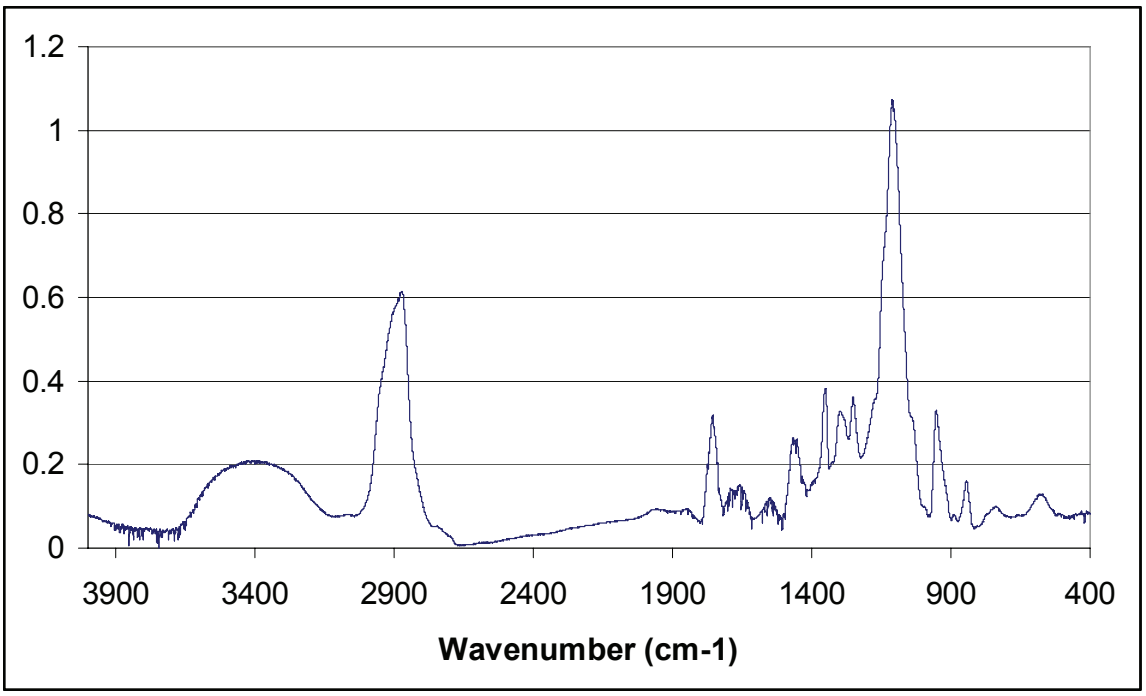

Figure 6.4: FTIR Spectrogram
FTIR Spectrogram of pegylated poly(glycolic acid). The peak at 1756 cm-1 shows the presence of an ester stretch due to glycolidyl moieties.

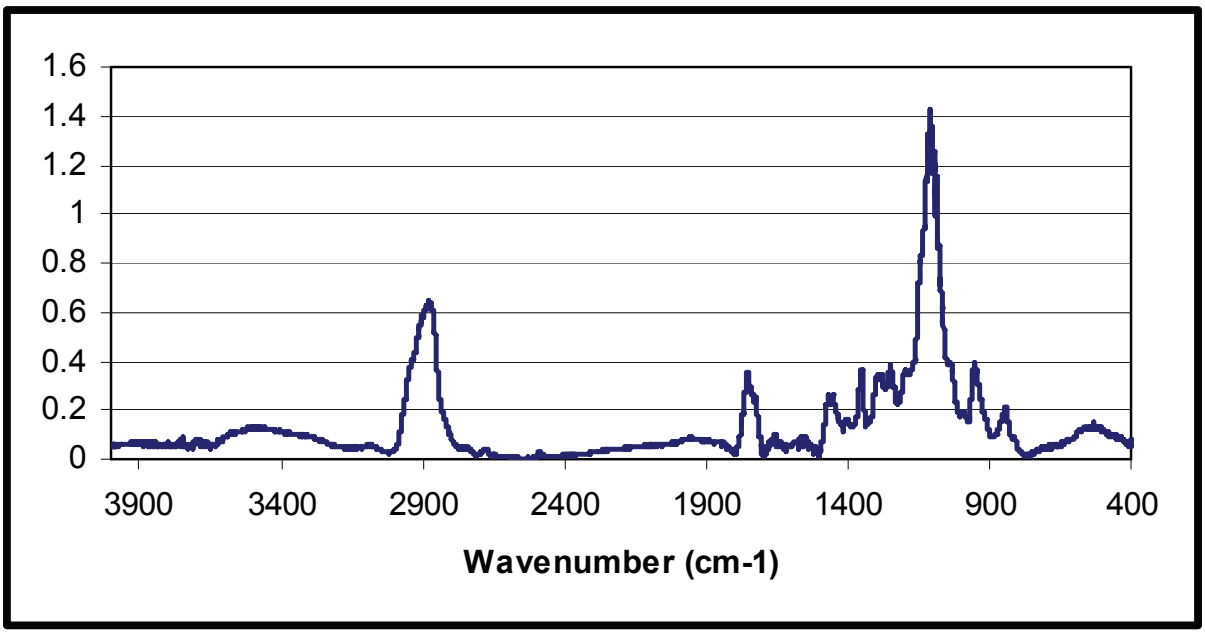

Figure 6.5: FTIR Spectrogram
FTIR spectrogram of pegylated poly(glycolic acid) diacrylate. The low absorbance at 3510 cm-1 is due to acrylation of the free hydroxyl group.

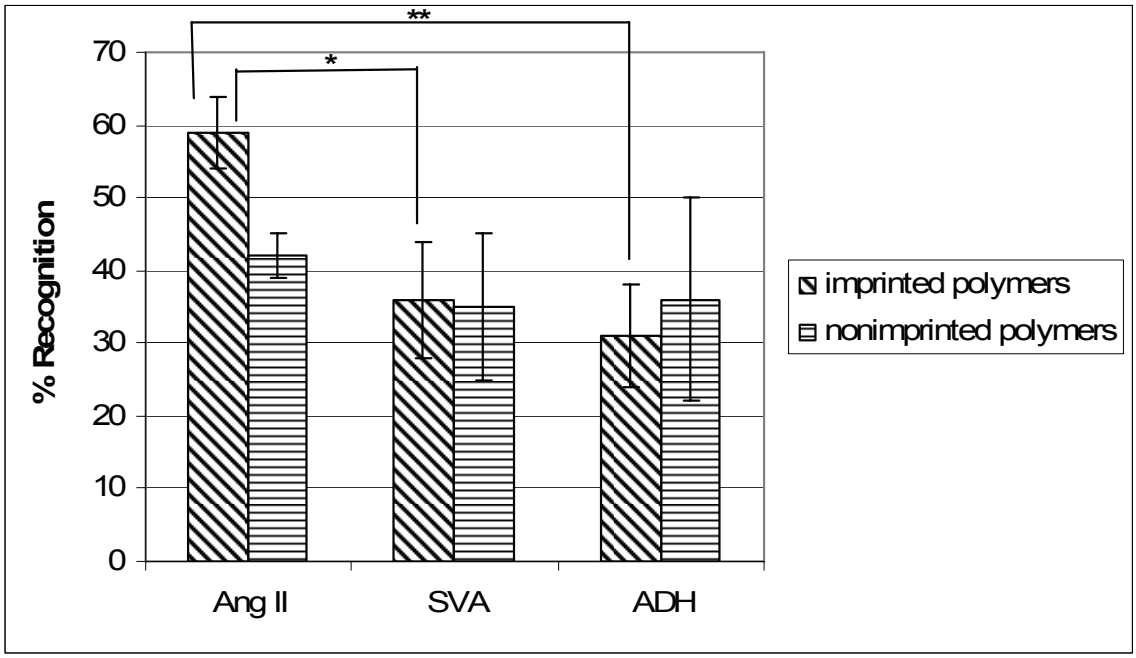

Figure 6.6: Specific Recognition of Degradable Network
Amount of peptide bound to polymer at a time point of 3hrs. n=3 * p=0.007, ** p=0.002

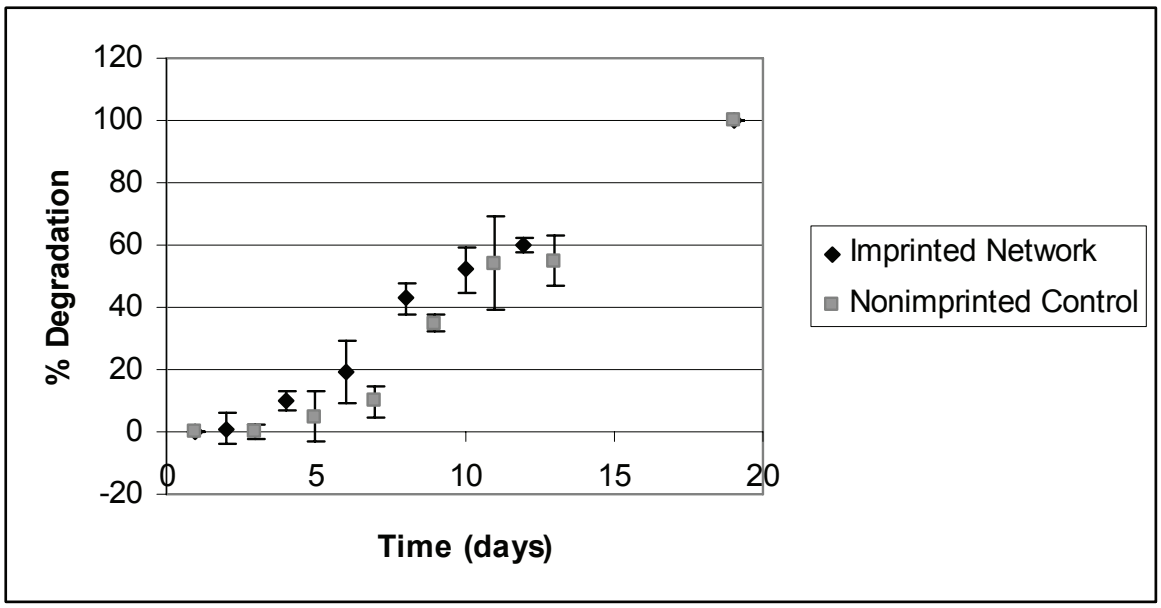

Figure 6.7: Percent Degradation over Time
Degradation kinetics for the imprinted network and the nonimprinted controls. Degradation took place in PBS at a pH of 7.4 at 37°C. n=3.

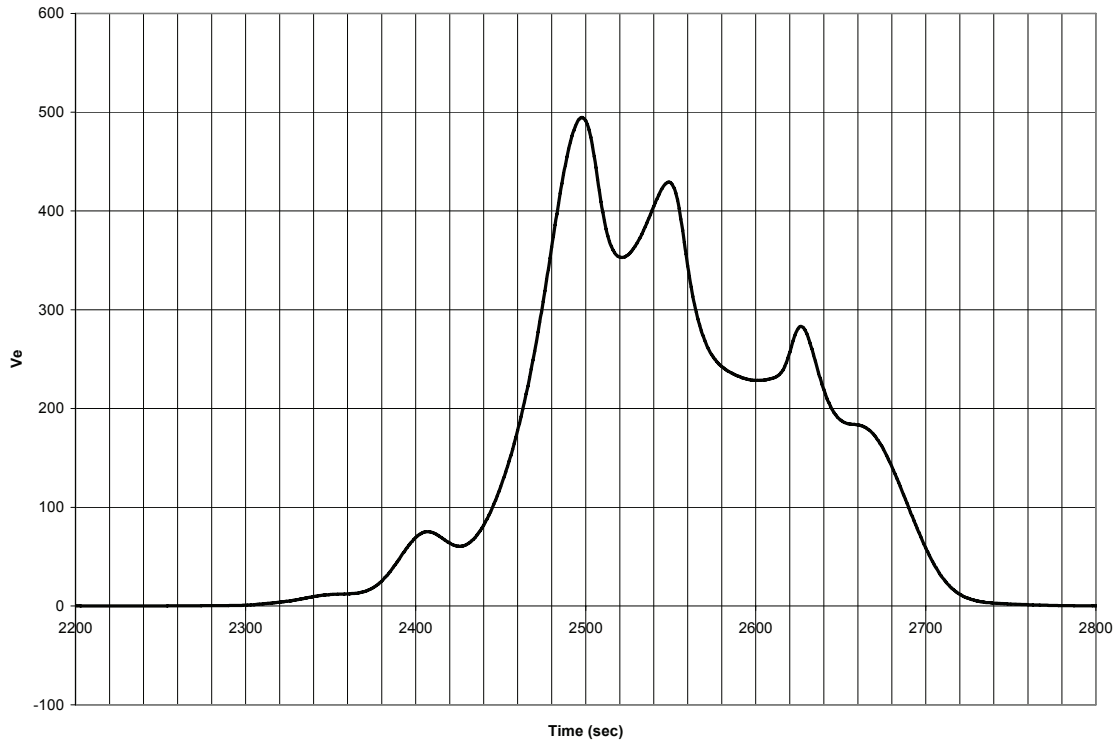

Figure 6.8: GPC Chromatograph
Elution peaks of degradation products. The large elution peak at 2496 sec corresponds to polymer peak.

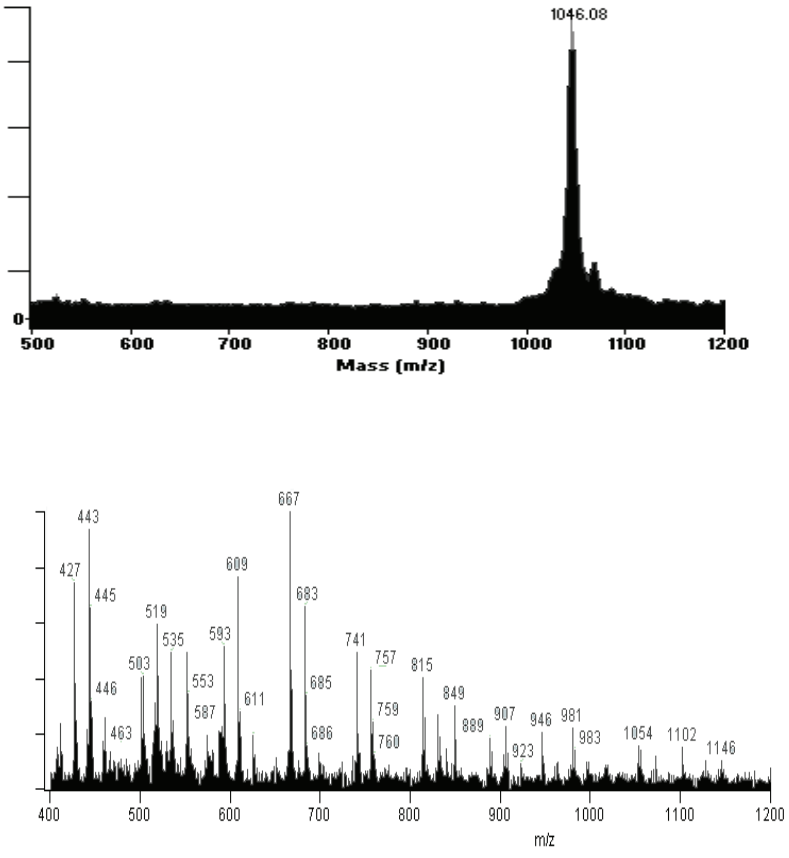

Figure 6.9: **ESI Mass Spectrograms**
ESI mass spectroscopy of pure angiotensin II (top) with 1046 peak and angiotensin II incubated in 70% glycolic acid for 24 hours (bottom).

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7 INVESTIGATION OF CYTOCOMPATIBILITY OF CONFIGURATIONAL BIOMIMETIC IMPRINTED POLYMERS SELECTIVE FOR ANGIOTENSIN II

7.1 Introduction

When synthetic biomaterials are placed into a physiological environment, it is essential that they are not cytotoxic, or cause more damage than good. Testing this is particularly important for polymeric devices since the starting materials such as monomers, initiators and solvents are often toxic or not biocompatible themselves.

Unreacted monomers and initiators are inherently toxic. However, in general terms when they become incorporated into a polymer network they are no longer dangerous. It must be noted though that during polymerization a 100% conversion is not achieved and unreacted monomers and initiator can become entrapped within the network. During use, these residues can migrate out and create cytotoxic effects. To minimize this, all biomaterials, including the polymer networks studied here, are washed twice a day for seven days. This has been shown to be a reasonable period to remove excess chemicals and unreacted monomers. [**1**] Also, since the polymerizations are free radical polymerizations, the start of each chain will incorporate the initiator into the polymer backbone also minimizing toxicity. In the present work, we performed a number of cytotoxicity and cellular studies using the newly developed, molecularly imprinted

hydrogels to investigate their biological behavior. Surprisingly enough, the literature on molecularly imprinted polymers does not typically include any biocompatibility studies.

7.2 Materials and Methods

7.2.1 Cell Culture

Cytocompatibility experiments were performed using an NIH/3T3 murine fibroblast cell line (#CRL-1658; American Type Culture Collection (ATCC), Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine and which was adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose (ATCC). Media was also supplemented with 10% fetal calf serum (ATCC), 1% non-essential amino acids ((Mediatech) Herndon, VA), 100 U/ml penicillin and 100 µg/ml streptomycin (Mediatech). The solution was then sterile filtered.

Cells were cultured according to ATCC protocols under strictly aseptic conditions. The frozen culture vile was rapidly warmed to 37°C. The cells were then transferred to a centrifuge tube containing 9.0 milliliters of DMEM. This mixture was spun at approximately 125 xg for 5 to 7 minutes. After aspirating the media, the resulting cell pellet was resuspended with the complete growth

medium and dispensed into a 75 cm^2 culture flask (T-75 flasks) (Corning, Corning, NY).

Before the cell pellet was added, the culture flask charged with the appropriate amount of complete growth medium was placed in the incubator for at least 15 minutes to establish the optimal pH range (7.0 – 7.6). The culture was then incubated at 37°C in a 95% humidified environment and 5% CO_2 atmosphere. During this time, the cell culture medium was changed approximately every two days.

When the cells reached 70% confluency, which typically occurred between 3 and 4 days after seeding, the cells were passaged. This was done by aspirating off the old medium. The flasks were then washed twice with 10 ml Dulbeccos Phophate Buffered Saline without $CaCl_2$ and $MgCl_2$ (DPBS, Sigma, St. Louis, MO) solution. The cells were then incubated with 5 ml of a 0.5% Trypsin/ 0.2% EDTA solution (Sigma) and incubated at 37°C for 5 minutes. Gentle agitation of the flasks caused the cells to detach at which time they could be counted and reseeded.

Upon cell detachment, 8 ml of DMEM were added, and the cells were gently aspirated via pipetting to break up cell clumps and detach remaining cells. A standard cell count was performed using a hemocytometer. The suspension

was then centrifuged at 150xg for 5 min. Upon completion, the remaining Trypsin/EDTA containing liquid was then aspirated off of the resulting cell pellet and replaced with fresh media to give a cell concentration of 3-5 X 10^3 cell/ cm^2. Appropriate aliquots of the cell suspension were then added to new T-75 cm^2 culture flasks. The culture flasks were incubated at 37°C under a 95% humidity and 5% CO_2 atmosphere.

7.2.2 Cytocompatibility Studies

Cytotoxicity studies were conducted in 96 well plates (Corning) when cells reached ~90% confluency or above (3-4 days). Cell studies were performed between passages 5-10. Media was refreshed 12 hours before the experiment. At the start of the experiment, growth medium was removed from each well.

CIBP and control microparticles were prepared as previously described. They were then added to wells at concentrations of 0.25 mg/mL, 0.5 mg/mL and 1.0 mg/mL suspended in DMEM. The CIBP formulations that were used were pegylated poly(acrylamide-co-methacrylic acid) with a crosslinker percentage of 40 mol% and a template to functional monomer ratio of 1:8. The formulation with the addition of the degradable crosslinker was also used. Prior to addition of the polymer microparticles, the pH of each suspension was adjusted to 7.4 with NaOH. Microparticles were then incubated with the cells for either 4 or 24 hours

at 37°C and 5% CO_2. The microparticle suspension was removed from each well, and the wells were rinsed three times with 200 µL Hanks Balanced Salt Solution (HBSS, Sigma) to remove microparticles and residual phenol red because this indicator is known to interfere with the absorbance readings of the assay.

Cytotoxicity was measured using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI). This method is a cellular metabolic assay used to measure NADPH production to give an accurate count of viable cells [**2**]. This assay contains a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyls)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS), and an electron coupling reagent, phenazine ethosulfate (PES). The MTS reagent is bioreduced by cells in culture to produce a colored formazan that is released into DMEM. It is believed the mechanism of reduction is achieved by NADPH contained in dehydrogenase enzymes from metabolically active cells (Figure 7.1). The absorbance of the formazan can then be read via a plate reader and corresponded to the viable number of cells (Figure 7.2).

A sample of 2 ml of assay reagent was suspended in 10 ml of HBSS. Then, 120 µL of the solution was added to each well and incubated with the cells for 1.5 hours at 37°C and 5% CO_2. A UV/vis microplate reader (Bio-Tek Synergy HT, Winooski, VT) was then used to determine the absorbance in each well at 490

nm. To determine cell viability, absorbance was compared to control wells that were not incubated with microparticles but only with HBSS and the CellTiter 96® reagent. Background absorbance of control wells containing only cells and HBSS was subtracted from the results. A 10% bleach solution was used as a negative control.

7.3 Results and Discussion

There was no significant decrease in cell viability when the various polymer formulations of the pegylated poly(acrylamide-co-methacrylic acid) network and its degradable counterpart were incubated with 3T3 fibroblasts. This was true for all concentrations of microparticles and at both time points of 4 h and 48 h. (Figure 7.3 and 7.4) Although there appeared to be some decrease in viability after 48 h, it was consistent with the control cells after normalization and was not considered to be a function of the polymer networks.

These results were encouraging but anticipated because the components of the networks have been previously shown to be biocompatible. PEG is considered a highly biocompatible polymer, and PGA has been FDA approved based on its biocompatibility. [**3**] The results also correlate well to previous results in our laboratory using similar polymer components on a different cell line. Those results showed that grafting methacrylic acid to PEG at a 1:1 molar feed in

microparticle formulations did not have cytotoxic effects when placed in contact with Caco-2 cell lines. This was at concentrations of less than 10 mg/ml [**4-7**].

These cytotoxicity results also suggest that potentially harmful network residues, such as any unreacted monomers and remaining photoinitiators in the polymer backbone, did not decrease cell viability. It can, therefore, be assumed that unreacted monomers have been adequately removed by washing steps. Photoinitiators, which have also been known to reduce cell viability, did not appear to have an effect either. Bryant et al. [**8**] and Williams et al. [**9**] concluded that the photoinitator Irgacure 184 often reduced cell viability in numerous different cell lines. However, these studies were conducted when the polymer was initiated and split into free radicals in the presence of cells. In the recognitive networks, the polymer is initiated before it is placed into contact with cells, therefore preventing them from being exposed to harmful free radicals.

7.4 Conclusions

Ultimately, it is the goal of this work to be able to place molecularly imprinted polymers into physiological environments. It is, therefore, essential to ensure that the materials are biocompatible. Although molecularly imprinted polymers have been well characterized and successfully used in many applications over the past several decades, there are no published results on their biocompatible nature.

These results are, therefore, some of the first of their kind to show that it is possible to create a recognitive network that does not exhibit any harmful biological side effects.

7.5 Figures

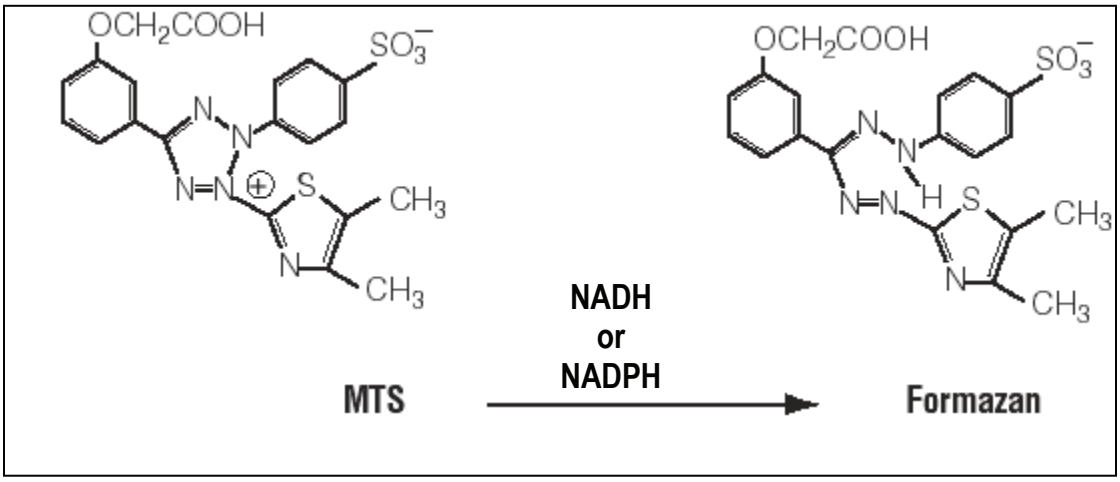

Figure 7.1: Chemical Structures of MTS Tetrazolium and Formazan

The CellTiter 96® AQueous One Solution Cell Proliferation Assay uses a MTS tetrazolium compound that is reduced to a colored formazan product by NADH or NADPH. The colorimetric change is directly proportional to the number of viable cells in culture. Adapted from Promega [**2**]

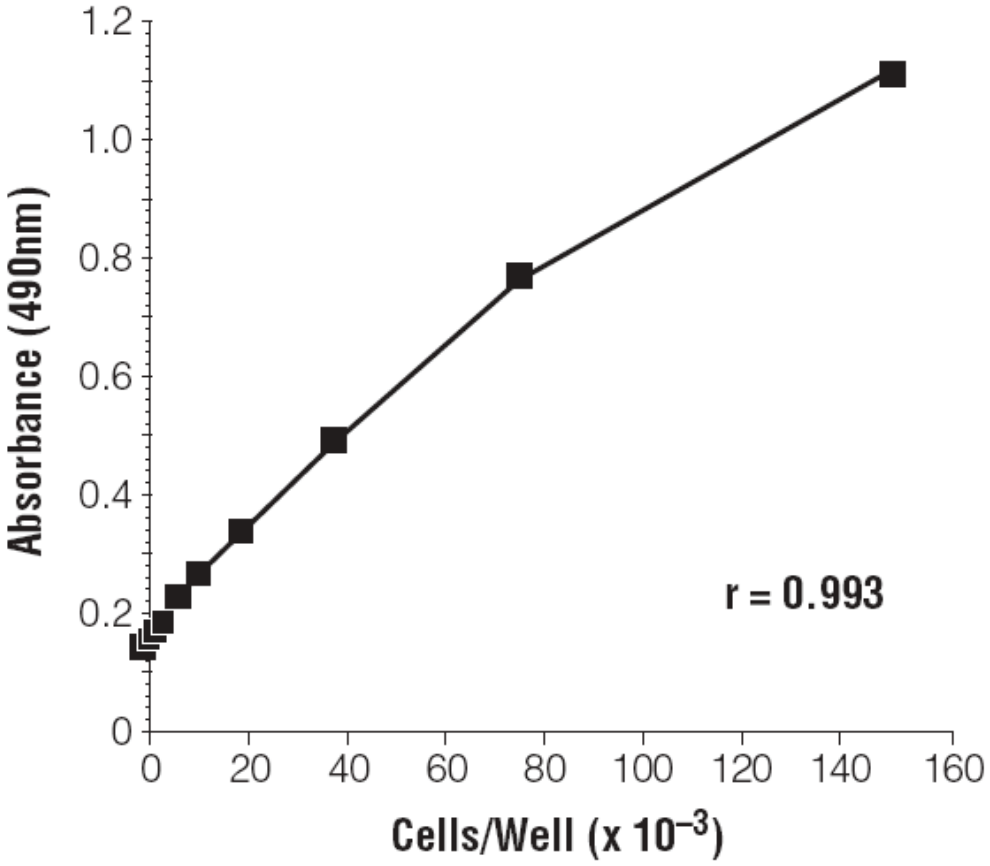

Figure 7.2: Corresponding Increase in Absorbance as an Indicator of Cell Number.

Figure reproduced from Promega [**2**].

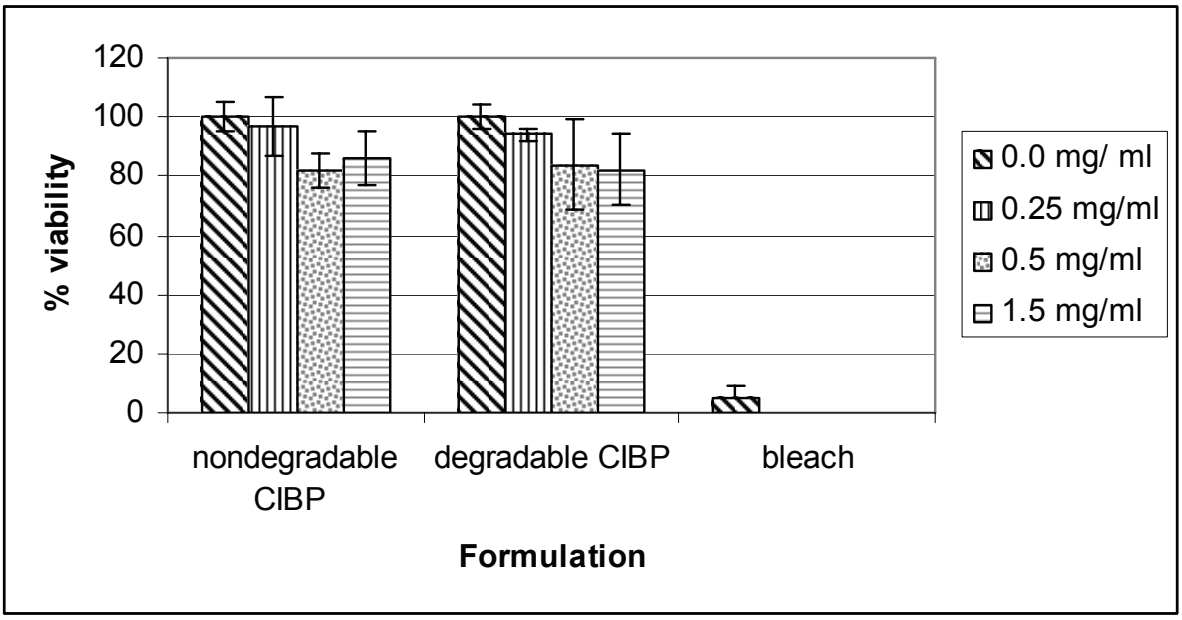

Figure 7.3: Cytotoxicity of imprinted polymers to 3T3 fibroblasts after 4h

Enzymatic activity of 3T3 cells after exposure to imprinted microparticles and 10% bleach. Results are expressed as the percentage of absorbance at 490 nm for a given microparticle concentration as compared to control wells containing only cells and HBSS. Cell viability was maintained across all concentrations and formulations. n=3

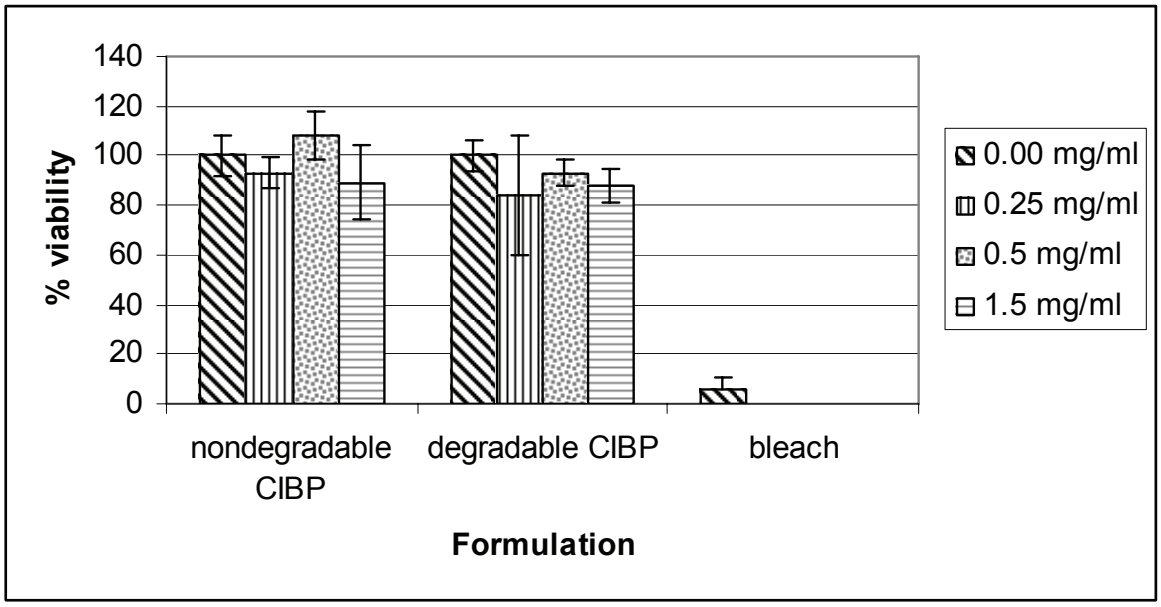

Figure 7.4: Cytotoxicity of imprinted polymers to 3T3 fibroblasts after 48h.

Enzymatic activity of 3T3 cells after exposure to increasing concentrations of imprinted microparticles and 10% bleach. Results are expressed as the percentage of absorbance at 490 nm for a given microparticle concentration as compared to control wells containing only cells and HBSS. Cell viability was maintained across all concentrations and formulations. n=3

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8 CONCLUSIONS

By investigating and understanding the molecular interactions of the human body, modern healthcare, which relies greatly upon diagnostic devices and drug delivery vehicles, can be greatly improved. In this field, there exists a great need for biocompatible materials which can be specifically tailored to interact with the shape and chemical properties of target molecules. To this end, the idea of molecular imprinting has emerged as a promising application in which synthetic materials are used in order to imitate the recognition capacity of natural systems towards specific ligands. MIP polymers have shown a significant ability to be both selective and sensitive toward their target molecules. The potential applications of MIP polymers are far reaching and extensive reviews have been published on their possible uses. This includes devices for affinity separations, antibody binding and enzyme mimetics, biomimetic sensors, clinical diagnostic devices and drug delivery vehicles [**1-8**].

Although imprinted polymers show great promise, the most successful MIP polymers have so far involved work containing smaller template molecules and ions. This is due primarily to the fact that large templates are less rigid. Ultimately, this can impair the formation of well-defined binding cavities during the imprinting process. Also, secondary and tertiary structures of large biomolecules are sometimes affected when exposed to the thermal or photo-

induced treatment required during the synthesis process. Rebinding is also more challenging for large molecules due to the inherent diffusion limitations through the polymer networks [**9-10**]. In biological systems, imprinted systems face several unique challenges. This includes the challenge of the impairment of imprinting induced recognition and selectivity in aqueous media due to the competition in hydrogen bonding between the solvent and template [**6**]. Also, if the network is placed in a physiological environment, it is important to ensure that the network is biocompatible. Ideally, the system would also be able to degrade.

Despite the challenges, the need exists to improve on currently available clinical diagnostic devices and DDS. The particular features of MIP polymers, which include their high affinity and selectivity compared to natural receptors, their unique stability that is superior to that of natural biomolecules, the simplicity of their preparation and the ease of adaptation to different practical applications, have made them the target of intense investigation [**7**].

This study shows one such investigation which was intended to present a possible solution to the many challenges posed by successfully imprinting larger biomolecules for physiological environments. An imprinted polymer which was able to recognize angiotensin II was effectively synthesized containing the functional monomers, methacrylic acid, acrylamide and PEG monomethacrylate. Various formulations and combinations of the monomers were studied in order to

determine which induced the best recognition of the peptide template. The synthesis process was carefully designed in order to create a successful recognition cavity. This included using DMSO as a solvent. Peptides and proteins maintain a similar hydrodynamic radius in DMSO as they do in water [**11**]. Therefore, the recognition cavities were able to maintain similar shape in an aqueous environment, minimizing negative effects during rebinding/recognition. Also, by including both acrylamide for hydrogen bonding and methacrylic acid for ionic bonding, these polymers were able to overcome the competition between the solvent and template and ultimately recognize the template molecule in an aqueous environment. The amount of crosslinking agent was also investigated in order to produce hydrogels that were able to maintain their physical integrity while at the same time allowing for sufficient diffusion of angiotensin II. The formulations were then tested in cytotoxic assays to determine their biocompatibility. In the end, the polymers, regardless of their specific formulation, did not exhibit any cytotoxic effects, indicating their biocompatible nature.

These materials were also studied via several characterization techniques to better understand their properties. SEM was used to study the porosity and microscopic features of the MIPs. The SEM images showed that the presence of the template protein had an effect on the polymerization, forming a more porous and structured polymer as compared to the controls. FTIR was used to confirm the presence of each of the monomers in the copolymer formulations as well as

to demonstrate the fact that the MIPs and control polymers are chemically identical. DSC was used to determine the glass transition temperatures of the crosslinked hydrogels and, from such information, the effective molecular weight between crosslinks. These values were compared to the theoretical molecular weight between crosslinks to ensure the validity of the observed T_g values.

This study was also the first of its kind to investigate the effects of adding a degradable crosslinker to the imprinted material. A poly(α–hyrdoxy ester) diacrylate crosslinker was successfully synthesized and incorporated into a network which remained recognitive and selective for angiotensin II. This degradable formulation was also found to be biocompatible in nature.

In the end, this study showed the successful creation of a biocompatible and biodegradable MIP polymer for a larger peptide. Key steps taken in the design and synthesis of the imprinted network allowed for binding in aqueous environments. Since angiotensin II is a model peptide and contains many attributes found in other peptides, these techniques can be readily expanded to additional peptides and/or proteins. By developing systems such as these, it is possible to broaden the scope of the molecular imprinting field, ultimately making it possible for imprinted systems to be successfully used in pharmaceutical treatments, drug delivery devices and tissue engineering constructs.

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